(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 391 516 A1

(12) EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
25.02.2004 Bulletin 2004/09

(21) Application number: **02722783.4**

(22) Date of filing: **25.04.2002**

(51) Int Cl.[7]: **C12N 15/57**

(86) International application number:
PCT/JP2002/004141

(87) International publication number:
WO 2002/088366 (07.11.2002 Gazette 2002/45)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: **25.04.2001 JP 2001128342**
27.07.2001 JP 2001227510
28.09.2001 JP 2001302977
25.01.2002 JP 2002017596

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(54) VON WILLEBRAND FACTOR (VWF)-CLEAVING ENZYME

(57) This invention is intended to isolate and identify a vWF-specific cleaving protease.

The vWF-specific cleaving protease cleaves a bond between residues Tyr 842 and Met 843 of vWF and comprises a polypeptide chain having Leu-Leu-Val-Ala-Val as a partial sequence, and more preferably comprises a polypeptide chain having the partial N-terminal amino acid sequence of a mature protein, Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val, and having a molecular weight of 105 to 160 kDa in SDS-PAGE under reducing or non-reducing conditions. Isolation and identification of this vWF-specific cleaving protease have led to the possibility of replacement therapy for patients having diseases resulting from a deficiency of the protease, such as thrombotic thrombocytopenic purpura.

EP 1 391 516 A1

Printed by Jouve, 75001 PARIS (FR)

Description

Technical Field

[0001] The present invention relates to a plasma protein related to the field of medical drugs. More particularly, the present invention relates to a protease that specifically cleaves von Willebrand factor (it may be hereafter referred to as "vWF"), which is associated with blood coagulation. The vWF-cleaving protease of the present invention enables replacement therapy for patients with diseases resulting from defects or decreases in this protease, such as thrombotic thrombocytopenic purpura (it may be hereafter referred to as "TTP"). In addition, the use thereof as a novel antiplatelet thrombotic agent is expected.

Background Art

[0002] vWF is produced in vascular endothelial cells or megakaryocytes, and is a blood coagulation factor in which a single subunit comprising 2,050 amino acid residues (monomers of about 250 kDa) are bound by an S-S bond to form a multimer structure (with a molecular weight of 500 to 20,000 kDa). The level thereof in the blood is about 10 μg/ml, and a high-molecular-weight factor generally has higher specific activity.

[0003] vWF has two major functions as a hemostatic factor. One of the functions is as a carrier protein wherein vWF binds to the blood coagulation factor VIII to stabilize it. Another function is to form platelet plug by adhering and agglomerating platelets on the vascular endothelial subcellular tissue of a damaged vascular wall.

[0004] Thrombotic thrombocytopenic purpura is a disease that causes platelet plug formation in somatic arterioles and blood capillaries throughout the whole body. In spite of recent advances in medical technology, the morbidity associated with this disease approximately tripled from 1971 to 1991. Pathologically, TTP is considered to result from vascular endothelial cytotoxicity or vascular platelet aggregation. Immunohistologically, a large amount of vWFs are recognized in the resulting platelet plugs, and vWF is considered to play a major role in causing them. A normal or high-molecular-weight vWF multimer structure is dominant in a TTP patient, and an unusually large vWF multimer (ULvWFM) or large vWF multimer (LvWFM) is deduced to play a major role in accelerating platelet aggregation or microthrombus formation under high shearing stress. In contrast, vWF was known to degrade at a position between residues Tyr 842 and Met 843 by the action of vWF-cleaving protease in the circulating blood of a healthy person under high shearing stress. Accordingly, TTP is considered to occur in the following manner. The protease activity in the plasma is lowered for some reason, and ULvWFM to LvWFM are increased to accelerate platelet aggregation. This forms platelet plugs in blood vessels.

[0005] Recently, Furlan et al. (Blood, vol. 87, 4223-4234: 1996, JP Patent Publication (Kohyo) No. 2000-508918) and Tsai et al. (Blood, vol. 87, 4235-4244: 1996) developed a method for assaying vWF-specific cleaving protease. In their report, this protease activity was actually lowered in TTP. The aforementioned authors reported that this enzyme was metalloprotease in the plasma and partially purified. However, they have not yet succeeded in the amino acid sequencing which would specify the protease. There have been no further developments since then.

Disclosure of the Invention

[0006] Up to the present, plasmapheresis therapy has been performed for treating patients who congenitally lack vWF-specific cleaving protease and patients who had acquired positive antibodies against this protease. Establishment of replacement therapy using purified products or a pure substance such as a recombinant gene product of the aforementioned protease is desired. Familial TTP patients congenitally lack vWF-specific cleaving protease, and non-familial TTP is caused by posteriori production of autoantibodies against the aforementioned protease. Accordingly, replacement therapy for this protease is preferable for familial TTP patients (plasma administration is actually performed), and removal of autoantibodies by plasmapheresis and substitution of this protease are necessary for non-familial TTP. Further, the use of this protease as a novel antiplatelet thrombotic agent can also be expected.

[0007] As mentioned above, however, Furlan et al. (Blood, vol. 87, 4223-4234: 1996, JP Patent Publication (Kohyo) No. 2000-508918) and Tsai et al. (Blood, vol. 87, 4235-4244: 1996) have suggested that the vWF-cleaving protease was metalloprotease in the plasma. It was reported to be partially purified, and concentrated 1,000- to 10,000-fold from the plasma in terms of its specific activity. Even under these conditions, there has been no advancement in the analysis of the properties of this protease, such as the amino acid sequence of its protein, over the period of roughly 5 years that has passed since then. No specific biological information has yet been obtained regarding this protease. As reported by Furlan et al., the protein of interest is supposed to be gigantic, and there may be various problems associated therewith. For example, diversified forms of this protease, such as various interacting molecules or cofactors, are expected. Based on the complexity of purification processes, deteriorated capacity of separation by nonspecific interaction during the purification step, and other factors, it is deduced to be very difficult to isolate and identify the protease

from a plasma faction by the purification process according to Furlan et al.

[0008] Under the above circumstances, the present inventors have conducted concentrated studies in order to isolate and identify the vWF-cleaving protease. As a result, they have succeeded in isolating and purifying the vWF-cleaving protease of interest, which had not yet been reported. Thus, they have succeeded in identifying an amino acid sequence of the mature protein and a gene encoding this amino acid sequence.

[0009] The vWF-cleaving protease of the present invention can cleave a bond between residues Tyr 842 and Met 843 of vWF. According to one embodiment, this protease has a molecular weight of 105 to 160 kDa or 160 to 250 kDa in SDS-PAGE under reducing or non-reducing conditions. It is comprised of a polypeptide chain having Leu-Leu-Val-Ala-Val as a partial sequence. More preferably, it is comprised of a polypeptide chain having the partial N-terminal amino acid sequence of a mature protein, i.e., Ala-Ala-Gly-Gly-De-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val. It is a novel substance characterized by the following properties.

1) vWF-cleaving activity

[0010] According to the N-terminal sequence analysis of the cleavage fragment, the protease of the present invention cleaves a peptide bond between residues Tyr 842 and Met 843.

2) Fractionation by gel filtration

[0011] When fractionation is performed by gel filtration chromatography using FI paste as a starting material, most activities are collected in a fraction with a molecular weight of 150 to 300 kDa. According to one embodiment of the present invention, an actually obtained active substance is found to have a molecular weight of about 105 to 160 kDa in electrophoresis. Accordingly, the protease of the present invention is a substance that is likely to form a dimer or the like or to bind to another molecule or a substance that can be easily degraded or can have a heterogeneous sugar chain added.

3) Ammonium sulfate precipitation

[0012] For example, when FI paste is used as a starting material, a large portion of this protease is recovered as a precipitation fraction from a roughly purified fraction with the use of 33% saturated ammonium sulfate.

4) SDS-PAGE

[0013] For example, the protease of the present invention derived from FI paste prepared from pooled human plasma or cryoprecipitate mainly has a molecular size of about 105 to 160 kDa determined by a molecular weight marker in SDS-PAGE. Based on the nucleic acid sequence as shown in SEQ ID NO: 15, when an amino acid sequence represented by a frame between an atg initiation codon at position 445 and a tga termination codon at position 4726 is expressed by gene recombination, there are some variations in molecular sizes depending on a host. However, a molecular size of about 160 to 250 kDa determined by a molecular weight marker is exhibited. This size is observed in the plasma of healthy humans and in that of some TTP patients. Several molecular species of this protease are present in human plasma, caused by the presence of alternative splicing products (SEQ ID NOs: 16 to 21) recognized at the time of gene cloning, differences in post-translational modification such as sugar chain addition, or degradation during purification. Further, this protease could be partially recovered in an active state after SDS-PAGE under non-reducing conditions.

5) Analysis of amino acid sequence

[0014] The amino acid sequence of the isolated polypeptide fragment was analyzed. This presented an example of a polypeptide chain having a sequence Leu-Leu-Val-Ala-Val as a partial amino acid sequence and a sequence Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val as a N-terminal amino acid sequence of a mature protein. Further, with current bioinformatics (BIOINFORMATICS: A Practical Guide to the Analysis of Genes and Proteins, edited by Andreas D. Baxevanis and B. F. Francis Ouellette), a nucleic acid sequence encoding the amino acid sequence was highly accurately identified by searching a database based on the aforementioned partial sequence. More specifically, the genome database was searched by the tblastn program. This identified a chromosome clone (AL158826) that is deduced to encode the protease of the present invention. Further, clones (AI346761 and AJ011374) that are deduced to be a part of the protease of interest and a part of the polypeptide to be encoded by the aforementioned genome were identified through collation with the Expressed Sequence Tag (EST) database. Based thereon, the amino acid sequence as shown in SEQ ID NO: 3 or 7 was identified as an active vWF-cleaving protease site.

[0015] GCT GCA GGC GGC ATC CTA CAC CTG GAG CTG CTG GTG GCC GTG, a sequence deduced from the genome, and more preferably CTG CTG GTG GCC GTG, a portion thereof, the transcriptome of which was confirmed by EST, was obtained. The obtained nucleotide sequence was analyzed, and motif analysis was carried out based on the deduced sequence. As a result, it was found to have a metalloprotease domain as a candidate for the protease of the present invention. Based on the above findings, it became possible to disclose a sequence of a polypeptide chain as a more specific example of the protease. Also, activities of proteases are generally known to vary depending on, for example, substitution, deletion, insertion, or introduction of point mutation into a portion of the amino acid sequence (Blood coagulation factor VII mutants, Soejima et al., JP Patent Publication (Kokai) No. 2001-61479 A). Similarly, the protease of the present invention,can be modified by, for example, deletion, substitution, or addition of one or several amino acids, to prepare optimized proteases.

[0016] The protease proteins were further mass-produced, and 29 amino acid sequences from the N-terminus were determined. These amino acid sequences are shown in SEQ ID NO: 8. This result is substantially the same as the sequence as shown in SEQ ID NO: 3 or 7 deduced by bioinformatics. Only one difference is that the amino acid 27th in SEQ ID NO: 3 or 7 was Glu while it was Arg according to the present analysis of the N-terminal sequence. This was considered to be a gene polymorphism. Thus, this protease was confirmed to be comprised of a polypeptide chain having the amino acid sequence as shown in SEQ ID NO: 3 or 7 at its N-terminus as a mature unit. A gene fragment encoding this protease was then cloned in the following manner.

[0017] Based on the nucleic acid sequence as shown in SEQ ID NO: 7, a sense primer (SEQ ID NO: 9) and an antisense primer (SEQ ID NO: 10) were prepared based on the nucleic acid sequence underlined in Fig. 9, and a gene sandwiched between these primers was amplified. This fragment was cloned, and the nucleotide sequence was then confirmed. This fragment was used as a probe for Northern blotting to analyze the site at which the protease gene was expressed. As a result, this protease gene was found to be expressed mainly in the liver. Accordingly, the human liver cDNA library was purchased, and a gene encoding this protease was identified using a rapid amplification of cDNA ends (RACE) technique. Based on these results, in the case of the largest sequence of approximately 5 kb of mRNA (cDNA) reaching the poly(A) addition site as shown in SEQ ID NO: 15 was identified.

[0018] Based on the amino acid sequence deduced from this gene sequence, this protease was deduced to have a preprosequence, and to belong to the disintegrin and metalloprotease (ADAM) family having a disintegrin-like domain, a metalloprotease domain, and the like, and particularly to the ADAM-TS family having a thrombospondin Type-1 (TSP-1) domain. Finally, including those having insertion or deletion in a part of the nucleic acid sequence, isoforms as shown in SEQ ID NOs: 16 to 21 having sequences as shown in SEQ ID NOs: 3 and 7 at the N-terminuses after the mature preprosequence has been cleaved were identified. Thus, the protease of the present invention should cleave vWF between residues Tyr 842 and Met 843 and should have the Leu-Leu-Val-Ala-Val sequence as a partial amino acid sequence.

[0019] The vWF-cleaving protease of the present invention can be generally prepared by the following process.

[0020] According to the present invention, a process for assaying the protease activity is characterized by the possibility of evaluating activity within a short period of time. According to the report by Furlan et al. (Blood, vol. 87, 4223-4234: 1996, JP Patent Publication (Kohyo) No. 2000-508918 A), activity is assayed by analyzing vWF-cleaving patterns by Western blotting using the anti-vWF antibody, and thus, it takes time to transfer the protease to a filter. More specifically, this process requires approximately at least 45 hours in total, i.e., 24 hours for the enzymatic reaction with a substrate vWF, 17 hours for electrophoresis, and 3 hours to transfer the protease to a filter, followed by detection using the anti-vWF antibody. In contrast, the present inventors completed activity assay in 18 hours in total, i.e., 16 hours for the enzymatic reaction with a substrate vWF, and 2 hours for electrophoresis and detection. This indicates that the time required for the assay can be reduced to one third or less of that required for the conventional assay. This can also shorten the time required for the purification process, and in turn can lower the degree of the protease to be inactivated. Accordingly, purification efficiency is improved compared with that attained by the method of Furlan et al., and as a result, the degree of purification is also enhanced.

[0021] Further, the starting material was examined using the aforementioned assay system. As a result, it was found that the protease activity was more concentrated in FI paste than in the cryoprecipitate that had been reported by Furlan et al. in the past. FI paste was used as a starting material, and the aforementioned rapid activity assay systems were combined. This enabled isolation and identification of the protease of interest. In a specific embodiment, a purification process combining gel filtration chromatography with ion exchange chromatography is employed, and the aforementioned activity assay system is also combined.

[0022] More specifically, FI paste is solubilized with a buffer, and the resultant is fractionated by gel filtration chromatography. The protease activity is fractionated at the elution region with a molecular weight of 150 to 300 kDa deduced from the size marker of gel filtration. Thereafter, the resultant is precipitated and concentrated using 33% saturated ammonium sulfate. This procedure is repeated three times in total. The active fraction obtained in the third gel filtration is pooled, and the resultant is subjected to dialysis at 4°C overnight with a buffer comprising 50 mM NaCl added to 50 mM Tris-HCl (pH 7.1). Thereafter, the dialysis product is subjected to anion exchange chromatography

(DEAE) and eluted stepwise with 0.25 M NaCl. The present inventors have conducted concentrated studies in order to find a process for isolating and identifying the protease of the present invention. As a result, they found that, surprisingly, the protease was recoverable as an active band after non-reducing SDS-PAGE. In order to achieve further mass production, the purified and concentrated fraction was applied to the Biophoresis utilizing the principle of SDS-PAGE. Thus, a fraction having vWF-cleaving activity was isolated from the electrophoresed fraction. According to the approximate calculation of the specific activity up to this phase, purification of about 30,000- to 100,000-fold was achieved. This procedure was efficiently and rapidly repeated several times, and thus, about 0.5 pmole of sample that is the current limit of the analysis of amino acid sequence was obtained. Thus, analysis of amino acid sequence became feasible. More specifically, a final step of separation and purification (Biophoresis) based on the principle of SDS-PAGE is important, and it is based on the findings as a result of concentrated studies, which had led to the completion of the present invention.

[0023] According to the report by Furlan et al., specific activity was improved by as much as about 10,000 times, although the protease was not substantially isolated or identified. This could be because of deactivation during purification or the difficulty of isolating and identifying molecules, which were gigantic proteins capable of interacting with various other proteins such as the protease of the present invention by a separation method utilizing various types of liquid chromatography. Further, the protease content in the plasma was deduced to be very small, and thus, it was necessary to await the establishment of the process according to the present invention. Furthermore, the use of this process enables the purification of recombinant genes.

[0024] Based on the findings of the present invention, peptides or proteins prepared from the obtained sequences are determined to be antigens. With the use thereof, a monoclonal antibody, a polyclonal antibody, or a humanized antibody thereof can be prepared by general immunization techniques (Current Protocols in Molecular Biology, Antibody Engineering: A PRACTICAL APPROACH, edited by J. McCAFFERTY et al. or ANTIBODY ENGINEERING second edition, edited by Carl A. K. BORREBAECK). Alternatively, an antibody that binds to the aforementioned protein can be prepared by antibody-producing techniques utilizing phage display (Phage Display of Peptides and Proteins: A Laboratory Manual, edited by Brian K. Kay et al., Antibody Engineering: A PRACTICAL APPROACH, edited by J. McCAFFERTY et al. or ANTIBODY ENGINEERING second edition, edited by Carl A. K. BORREBAECK). Alternatively, based on these techniques, a neutralizing antibody acting against the protease activity or a simple binding antibody can be isolated from a specimen from a TTP patient who has an autoantibody positive against this protease. These antibodies can be applied to diagnosis and therapy of diseases such as TTP.

[0025] Based on the obtained genome or EST sequence, cDNA or a genomic gene encoding the protease of the present invention can be cloned by a common technique (Molecular Cloning, 2nd edition). Further, bioinformatics techniques (BIOINFORMATICS: A Practical Guide to the Analysis of Genes and Proteins, edited by Andreas D. Baxevanis and B. F. Francis Ouellette) enable cloning of the proteins of other animal species that are homologous thereto, and the resultant gene is fractured by a common technique (for example, Gene Targeting: A Practical Approach, First Edition, edited by A. L. Joyner, Teratocarcinomas and embryonic stem cell a practical approach) to produce TTP-like animal models. In particular, the identification of the gene sequence encoding the protein derived from a mouse enables the production of a knockout mouse having this gene. Thus, a disease mouse model of congenital TTP or the like can be prepared.

[0026] In accordance with a common technique (for example, J. Sambrook et al., Molecular Cloning, 2nd edition, or CURRENT PROTOCOLS IN MOLECULAR BIOLOGY), these genes are incorporated into a suitable expression vector, the resultant is transformed into a suitable host cell, and the gene recombinant product of the protease can be thus prepared. In this case, the gene to be incorporated is not necessarily the one that encoded the entire region of the protein. It also includes a partial expression of the protein as defined by a domain depending on its usage.

[0027] For example, the polynucleotide according to the present invention is introduced into a host cell using a conventional technique such as transduction, transfection, or transformation. The polynucleotide is introduced solely or together with another polynucleotide. Another polynucleotide is introduced independently, simultaneously, or in combination with the polynucleotide of the present invention.

[0028] For example, the polynucleotide of the present invention is transfected in a host cell, such as a mammalian animal cell, by a standard technique for simultaneous transfection and selection using another polynucleotide encoding a selection marker. In this case, the polynucleotide would be generally stably incorporated in the genome of the host cell.

[0029] Alternatively, the polynucleotide may be bound to a vector comprising a selection marker for multiplication in a host. A vector construct is introduced to a host cell by the aforementioned technique. In general, a plasmid vector is introduced as DNA of a precipitate, such as a calcium phosphate precipitate, or a complex with a charged lipid. Electroporation is also employed for introducing the polynucleotide into a host. When the vector is a virus, this virus is packaged *in vitro* or introduced into a packaging cell, thereby introducing the packaged virus into a cell.

[0030] Extensive techniques that are suitable for producing a polynucleotide and introducing the resulting polynucleotide to a cell in accordance with this embodiment of the present invention are known and common in the art. Such techniques are described in Sambrook et al. (aforementioned), and this document explains a variety of standard 'ex-

perimental manuals describing the aforementioned techniques in detail. In respect of this embodiment of the present invention, the vector is, for example, a plasmid vector, a single- or double-stranded phage vector, or a single- or double-stranded RNA or DNA viral vector. Such a vector is introduced into a cell as a polynucleotide, and preferably as DNA by a common technique for the introduction of DNA or RNA into a cell. When the vector is a phage or virus, the vector is preferably introduced to the cell as a packaged or sealed virus by a known technique for infection and transduction. A viral vector may be of a replication-competent or defective type.

[0031] A preferable vector is a vector which expresses the polynucleotide or polypeptide of the present invention in points. In general, such a vector comprises a cis-action control region that is effective for the expression in a host operably bound to the polynucleotide to be expressed. When a suitable trans-action factor (for example, a group of proteases involved with the post-translational processing such as signal peptidase or Furin) is introduced in a host cell, it is supplied by a host, a complementary vector, or the vector itself.

[0032] In a preferable embodiment, a vector provides specific expression. Such specific expression is an inducible one or realized only in a certain type of cell. Alternatively, it is an inducible and cell-specific expression. A particularly preferable inducible vector can induce expression by an easily operable environmental factor such as temperature or a nutritional additive. Various vectors suitable for this embodiment including a construction for the use in prokaryotic and eukaryotic cell hosts and an inducible expression vector are known, and persons skilled in the art can commonly use them.

[0033] A genetically engineered host cell can be cultured in general nutrient medium, and it is modified to be particularly suitable for activation of promoter, selection of transformant, or amplification of a gene. In general, it would be obvious to persons skilled in the art that conventional culture conditions such as temperature or pH level for host cells selected for the expression are suitable for the expression of the polypeptide of the invention.

[0034] A wide variety of expression vectors can be used for expressing the polypeptide of the present invention. Examples of these vectors include chromosome, episome, and virus-derived vectors. These vectors are derived from bacterial plasmid, bacteriophage, yeast episome, yeast chromosome element, or viruses such as baculovirus, papovavirus such as simian virus 40 (SV40), vaccinia virus, adenovirus, fowlpox virus, pseudorabies virus, or retrovirus. A vector derived from a combination of the aforementioned, for example, a vector derived from plasmid and bacteriophage gene element, more specifically, a cosmid or phagemid, may also be used. They are used for the expression in accordance with this embodiment of the present invention. In general, since polypeptides were expressed in hosts, any vector that is suitable for maintaining, multiplying, or expressing a polynucleotide can be used for the expression according to the aforementioned embodiment. A suitable DNA sequence is inserted into a vector by various conventional techniques. In general, a DNA sequence for expression is bound to an expression vector by cleavage of a DNA sequence and an expression vector having 1 or more restriction endonucleases, and a restriction fragment is then bound together using T4 DNA ligase. Restriction and ligation techniques that can be used for the above purpose are known and common to persons skilled in the art. With regard thereto, Sambrook et al. (aforementioned) very precisely describe another suitable method for constructing an expression vector utilizing another technique known and common to persons skilled in the art.

[0035] A DNA sequence in the expression vector is operably bound to, for example, a suitable expression-regulating sequence including a promoter to orient the mRNA transcription. A few examples of known representative promoters are the phage lambda PL promoter, *E. coli* lac, trp, trc, and tac promoters, SV40 early and late promoters, and the retrovirus LTR promoter. Many promoters that are not described are suitable for the use according to the embodiment of the present invention, known, and more easily used as described in the examples of the present invention. In general, an expression construct comprises a ribosome binding site for translation in a transcription initiation or termination site or a transcribed domain. The coding region of the mature transcript that was expressed by the construct comprises the initiation AUG at the initiation and termination codons located substantially at the terminus of polypeptide to be translated. In addition, the construct comprises a regulator region that regulates and induces the expression. In general, such a region is activated through the regulation of the repressor binding site, transcription of an enhancer, or the like in accordance with various conventional methods.

[0036] Vectors for multiplication and expression include selection markers. Such markers are suitable for multiplication, or they comprise additional markers for the above-stated purpose. The expression vector preferably comprises one or more selection marker genes to provide phenotypic traits for the purpose of selecting the transformed host cell. A preferable marker includes dihydrofolate reductase- or neomycin-resistance with regard to eukaryotic cell culture. It has tetracycline- or ampicillin-resistance with regard to *E. coli* and other bacterial cultures. A suitable vector comprising a DNA sequence and a suitable promoter or regulatory sequence as described herein are introduced to a suitable host by various suitable known techniques for the expression of the polypeptide of interest.

[0037] Representative examples of suitable hosts include: bacterial cells such as *E. coli, Streptomyces*, and *Salmonella typhimurium*; fungal cells such as a yeast cell; insect cells such as drosophila S2 and Spodoptera Sf9 cells; and adhesive or floating animal or plant cells such as CHO, COS, Bowes melanoma cells, and SP2/0. Various hosts for expression constructs are known, and persons skilled in the art can easily select a host for expressing polypeptides

in accordance with this embodiment based on the disclosure of the present invention.

[0038] More specifically, the present invention includes a recombinant construct, such as an expression construct comprising one or more sequences as mentioned above. The construct is a vector, such as a plasmid or viral vector comprising the sequence of the present invention inserted therein. The sequence is inserted in a positive or negative direction. In a preferable specific example thereof, the construct further has a regulatory sequence comprising a promoter or the like that is operably bound to the sequence. Various suitable vectors and promoters are known to persons skilled in the art, and there are many commercially available vectors that are suitably used in the present invention.

[0039] Commercially available vectors are exemplified below. Vectors that are preferably used for bacteria are pQE70, pQE60, and pQE-9 (Qiagen); pBS vector, PhageScript vector, Bluescript vector, pNH8A, pNH16a, pNH18A, and pNH46A (Stratagene); and ptrc99a, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia). Examples of preferable eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1, and pSG (Stratagene) and pSVK3, pBPV, pMSG, and pSVL (Pharmacia). These vectors are commercially available for persons skilled in the art to be used in accordance with the embodiment of the present invention, and they are merely a list of known vectors. For example, other plasmids or vectors suitable for introducing, maintaining, multiplying, or expressing the polynucleotide or polypeptide of the present invention can also be used in hosts in accordance with this embodiment of the present invention.

[0040] A promoter region can be selected from a gene of interest using a vector comprising, for example, a candidate promoter fragment, i.e., a reporter transcription unit lacking a promoter region such as a chloramphenicol acetyl transferase (CAT) transcription unit located downstream of restriction sites for introducing promoter-containing fragments. As known to the public, the introduction of the promoter-containing fragment into the vector at the restriction site located upstream of the cat gene generates CAT activity that can be detected by standard CAT assay. A vector that is suitable for this purpose is known and readily available. Examples of such vectors are pKK232-8 and pCM7. Accordingly, the promoter for expressing the polynucleotide of the present invention includes not only a readily available known promoter but also a promoter that can be readily obtained using a reporter gene in accordance with the aforementioned technique.

[0041] Among them, according to the present invention, examples of known bacterial promoters that are suitably used to express polynucleotides and polypeptides are *E. coli* lacI and lacZ promoters, T3 and T7 promoters, gpt promoter, lambda PR and PL promoters, and trp and trc promoters. Examples of suitable known eukaryotic promoters include the Cytomegalovirus (CMV) immediate promoter, the HSV thymidine kinase promoter, early and late SV40 promoters, a retrovirus LTR promoter such as the Rous sarcoma virus (RoSV) promoter, and a metallothionein promoter such as the metallothionein-I promoter.

[0042] Selection of a vector and a promoter suitable for expression in a host cell is a known technique. Techniques necessary for the construction of expression vectors, introduction of a vector in a host cell, and expression in a host are common in the art. The present invention also relates to a host cell having the aforementioned construct. A host cell can be a higher eukaryotic cell such as a mammalian animal cell, a lower eukaryotic cell such as a yeast cell, or a prokaryotic cell such as a bacterial cell.

[0043] The construct can be introduced in a host cell by calcium phosphate transfection, DEAE-dextran-mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. These methods are described in a variety of standard laboratory manuals, such as a book by Sambrook et al.

[0044] The construct in a host cell can be used by a conventional method, and it produces a gene product encoded by a recombinant sequence. Alternatively, a partial polypeptide of the present invention can be synthesized using a general peptide synthesizer. A mature protein can be expressed under the control of a suitable promoter in a mammalian animal, yeast, bacterial, or other cell. Also, such a protein can be produced in a cell-free translation system with the use of RNA derived from the DNA construct of the present invention. Suitable cloning and expression vectors for prokaryotic and eukaryotic hosts are described in Sambrook et al (aforementioned).

[0045] In general, a recombinant expression vector comprises: a replication origin; a promoter derived from a highly expressed gene to orient the transcription of a downstream structural sequence; and a selection marker for bringing the cell into contact with a vector and isolating the vector-containing cell. A suitable promoter can be induced from a gene encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, and heat shock protein. A selection marker includes *E. coli* ampicillin-resistant gene and *S. cerevisiae* trp1 gene.

[0046] Transcription of DNA encoding the polypeptide of the present invention using a higher eukaryotic cell may be enhanced by inserting an enhancer sequence in a vector. The enhancer is generally a cis-acting element for DNA for enhancing the promoter transcription activity in the predetermined host cell. Examples of an enhancer include the SV40 enhancer, the Cytomegalovirus early promoter/enhancer, the polyoma enhancer behind the replication origin, the β-actin enhancer, and the adenovirus enhancer.

[0047] The polynucleotide of the present invention encoding a heterologous structural sequence of the polypeptide of the present invention is generally inserted in a vector by standard techniques in such a manner that it is operably bound to the expression promoter. The transcription initiation site of the polypeptide is suitably located at the 5' site of the ribosome binding site. The ribosome binding site is 5' relative to AUG that initiates the translation of a polypeptide to be expressed. In general, an initiation codon starts from AUG and another open reading frame located between the

ribosome binding site and initiation AUG is not present. The termination codon is generally present at the terminus of the polypeptide, and the adenylation signal and the terminator are suitably located at the 3' end of the transcription region.

[0048] Regarding the secretion of the translated protein in the ER lumen, in the cytoplasm, or to the extracellular environment, a suitable secretion signal is incorporated in the expressed polypeptide. The signal may be endogenous or heterologous to the polypeptide.

[0049] Further, a prosequence subsequent to the signal sequence may be endogenous or heterologous (e.g., a preprosequence of another metalloprotease).

[0050] The polypeptide is expressed in a modified form such as a fusion protein, and it includes not only a secretion signal but also an additional heterologous functional region. Accordingly, an additional amino acid, especially a charged amino acid region, or the like, is added to the polypeptide to improve stability and storage stability in the host cell during purification or subsequent operation and storage. Alternatively, a given region may be added to the polypeptide to accelerate the purification. This type of region may be removed before the final preparation of polypeptides. Induction of secretion or excretion, stability improvement, or facilitation of purification with the addition of a peptide portion to the polypeptide is a technique common and known in the art.

[0051] Examples of prokaryotic hosts that are suitable for multiplying, maintaining, or expressing the polynucleotide or polypeptide of the present invention include *E. coli, Bacillus subtilis*, and *Salmonella typhimurium*. Various types of *Pseudomonas, Streptomyces*, and *Staphylococcus* are suitable hosts in this respect. Furthermore, various other types of hosts known to persons skilled in the art can be also used. Representative examples of expression vectors that are useful for bacterial applications include, but are not limited to, the replication origin of bacteria derived from commercially available plasmid including a selectable marker and a gene element of a known cloning vector pBR322 (ATCC 37017). Examples of such commercially available vectors include pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, Wisconsin, USA). These pBR322 (main chain) sections are combined with a suitable promoter and structural sequences to be expressed.

[0052] Host cells are suitably transformed and multiplied to the optimal cell concentration. Thereafter, the selected promoter is induced by a suitable means (e.g., temperature shifting or chemical inducer), and cells are further cultured. Typically, cells are collected by centrifugation and fractured by a physical or chemical means. The resulting crude extract is further purified. Microbial cells used for the protein expression can be fractured by any convenient means selected from a freezing-thawing cycle, ultrasonication, mechanical fracture, and the use of a cytolytic agent. These methods are known to persons skilled in the art.

[0053] Various cell lines for mammalian animal cell culture can be also used for the expression. An example of a cell line for mammalian animal expression includes a monkey kidney fibroblast COS-6 cell described in Gluzman et al., Cell 23: 175 (1981). Examples of other cells that are capable of expressing compatible vectors include C127, 3T3, CHO, HeLa, human kidney 293, and BHK cells. Further, a floating myeloma cell line such as SP2/0 can be also used.

[0054] A mammalian animal expression vector comprises a replication origin, a suitable promoter and enhancer, a necessary ribosome binding site, a polyadenylation site, splice donor and acceptor sites, a transcription termination sequence, and a 5' franking untranscribed sequence necessary for expression. DNA sequences derived from the SV40 splice site and the SV40 polyadenylation site are used for the non-transformed or transcribed gene element of interest. An example thereof is a CAG expression vector (H. Niwa et al., Gene, 108, 193-199 (1991)).

[0055] Based on the gene sequence of the above protease, a probe, primer, or antisense is designed by a common technique. The antisense technique can be used for controlling gene expression by the use of antisense DNA or RNA or the formation of a triple helix. This technique is described in, for example, Okano, J., Neurochem., 56: 560 (1991); OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION, CRC Press, Boca Raton, FL (1988). The triple helix formation is examined in, for example, Lee et al., Nucleic Acids Research 6: 3073 (1979); Cooney et al., Science 241: 456 (1988); and Dervan et al., Science 251: 1360 (1991). The method is based on the polynucleotide bond with complementary DNA or RNA. This enables the gene diagnosis or gene therapy.

[0056] For example, cells obtained from a patient are subjected to *ex vivo* genetic engineering using a polynucleotide such as polypeptide-encoding DNA or RNA. The resulting cells are then supplied to patients who should be treated with polypeptides. For example, cells can be subjected to *ex vivo* genetic engineering using a retrovirus plasmid vector comprising RNA encoding the polypeptide of the present invention. Such a technique is known in the art, and the use thereof in the present invention is obvious according to the description given herein. Similarly, cells are subjected to *in vitro* genetic engineering in accordance with a conventional process in respect of *in vivo* polypeptide expression. For example, the polynucleotide of the present invention is genetically engineered for expression in the replication-deficient retrovirus vector as mentioned above. Subsequently, the retrovirus expression construct is isolated, introduced to a packaging cell, and transduced using a retrovirus plasmid vector comprising RNA encoding the polypeptide of the present invention. Thus, the packaging cell produces infectious viral particles having a control gene. These producer cells are subjected to *in vitro* genetic engineering and then administered to patients to allow polypeptides to be expressed *in vivo*. This administration method and other methods for administering polypeptides according to the present

invention would be clearly understood by persons skilled in the art based on the teaching of the present invention.

[0057] Examples of the aforementioned retrovirus, from which the retrovirus plasmid vector is derived, include, but are not limited to, Moloney murine leukemia virus, spleen necrosis virus, Rous sarcoma virus, Harvey sarcoma virus, avian leukosis virus, gibbon leukemia virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. This type of vector comprises one or more promoters to express polypeptides. Examples of suitable promoters that can be used include, but are not limited to, retrovirus LTR, SV40 promoter, CMV promoter described in Miller et al., Biotechniques 7: 980-990 (1989), and other promoters (e.g., cell promoters such as a eukaryotic cell promoter including, but not limited to, histone, RNA polymerase III, and β-actin promoter). Examples of other viral promoters that can be used include, but are not limited to, adenovirus promoter, thymidine kinase (TK) promoter, and B19 Parvovirus promoter. Persons skilled in the art can readily select a suitable promoter based on the teaching of the present invention.

[0058] A nucleic acid sequence that encodes the polypeptide of the present invention is under the control of a suitable promoter. Examples of suitable promoters that can be used include, but are not limited to, adenovirus promoter such as adenovirus major late promoter, heterologous promoter such as CMV promoter, respiratory syncytial virus (RSV) promoter, inducible promoter such as MMT promoter or metallothionein promoter, heat shock promoter, albumin promoter, ApoAI promoter, human globin promoter, viral thymidine kinase promoter such as herpes simplex thymidine kinase promoter, retrovirus LTR including the aforementioned modified retrovirus LTR, β-actin promoter, and human growth hormone promoter. A promoter may be of a native type that controls the gene encoding polypeptides. A retrovirus plasmid vector is used to transduce the packaging cell line to form a producer cell line.

[0059] Examples of packaging cells to be transfected include, but are not limited to, PE501, PA317, Y-2, Y-AM, PA12, T19-14X, VT-19-17-H2, YCRE, YCRIP, GP+E-86, GP+envAm12, and the DAN cell line described in Miller, Human Gene Therapy 1: pp. 5-14 (1990).

[0060] A vector is transduced in a packaging cell by a means known in the art. Examples of such means include, but are not limited to, electroporation, the use of a liposome, and $CaPO_4$ precipitation. Alternatively, a retrovirus plasmid vector is sealed in a liposome or bound to a lipid to be administered to a host. A producer cell line produces infectious retrovirus vector particles comprising nucleic acid sequences encoding polypeptides. Such retrovirus vector particles are used to transduce eukaryotic cells *in vitro* or *in vivo*.

[0061] The transduced eukaryotic cells express nucleic acid sequences encoding polypeptides. Examples of eukaryotic cells that may be transduced include, but are not limited to, germinal stem cells, embryonal carcinoma cells, hematopoietic stem cells, hepatic cells, fibroblasts, sarcoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

[0062] The protease of the present invention, an antibody against this protease, an antagonist of this protease, an inhibitor, an agonist, an activity modifier, or the like can be diluted with physiological saline, buffer, or the like to prepare a formulation. Thus, a pharmaceutical composition can be obtained. The pH value of the formulation is preferably between acidulous and neutral: close to the pH level of body fluid. The lower limit thereof is preferably between 5.0 and 6.4, and the upper limit is preferably between 6.4 and 7.4. Alternatively, the formulation can be provided in a state that allows storage for a long period of time, e.g., in a lyophilized state. In such a case, the formulation can be used by being dissolved in water, physiological saline, buffer, or the like at a desired concentration level at the time of use.

[0063] The formulation of the present invention may comprise a pharmacologically acceptable additive, such as a carrier, excipient, or diluent that is commonly used for pharmaceuticals, a stabilizer, or pharmaceutically necessary ingredients. Examples of a stabilizer include monosaccharides such as glucose, disaccharides such as saccharose and maltose, sugar alcohols such as mannitol and sorbitol, neutral salts such as sodium chloride, amino acids such as glycine, nonionic surfactants such as polyethylene glycol, polyoxyethylene and polyoxypropylene copolymers (Pluronic), polyoxyethylene sorbitan fatty acid ester (Tween), and human albumin. Addition thereof in amounts of about 1 to 10 w/v% is preferable.

[0064] An effective amount of the pharmaceutical composition of the present invention can be administered by, for example, intravenous injection, intramascular injection, or hypodermic injection in one or several separate dosages. The dosage varies depending on symptom, age, body weight, or other factors, and it is preferably 0.001 mg to 100 mg per dose.

[0065] Also, sense or antisense DNA encoding the protease of the present invention can be similarly prepared in a formulation to obtain a pharmaceutical composition.

[0066] Further, the present invention includes methods for inhibiting platelet plug formation involved with heart infarction or brain infarction, methods for inhibiting arteriosclerosis, methods for preventing restenosis, reembolization, or infarction involved with PTCA, methods for preventing reembolization involved with PTCR, and methods for preventing platelet plug formation caused by HUS or O-157 through the administration of the peptide, protein, and DNA of the present invention. Furthermore, the present invention includes the use of the peptide, protein, and DNA of the present invention in the production of pharmaceuticals for inhibiting platelet plug formation involved with heart infarction or brain infarction, pharmaceuticals for inhibiting arteriosclerosis, pharmaceuticals for preventing restenosis, reembol-

ization, or infarction involved with PTCA, pharmaceuticals for preventing reembolization involved with PTCR, and pharmaceuticals for preventing platelet plug formation caused by HUS or O-157.

[0067] The peptide or protein of the present invention is used as a leading substance for amino acid modification. This enables the preparation of a molecule having activity that is different from that of the protease of the present invention. An example thereof is a variant molecule that can be obtained by preparing an antagonist, which is obtained by preparing a variant deactivated through amino acid substitution between an amino acid residue located around the active center in the metalloprotease domain and another amino acid, separating a molecule recognition site from a catalytic site, or varying one or both of these sites.

[0068] The use of an evaluation system for the vWF-cleaving activity described herein enables the production of an antagonist/agonist. For example, an effective antagonist can be a small organic molecule, a peptide, or a polypeptide. An example thereof is an antibody that is bound to the polypeptide of the present invention, thereby inhibiting or eliminating its activity.

[0069] Similarly, the use of the aforementioned evaluation system for vWF-cleaving activity enables the screening for a compound that is capable of cleaving vWF. In such a case, the cleaving activity of the test compound may be evaluated using the aforementioned evaluation system.

Brief Description of the Drawings

[0070]

Fig. 1 is a diagram showing the vWF multimer structure and the point cleaved by the vWF-cleaving protease.

Fig. 2 is a photograph showing the result of vWF multimer analysis (agarose electrophoresis).

Fig. 3 is a photograph showing the result of SDS-PAGE (5% gel) for analyzing the vWF-cleaving activity of each plasma fraction under reducing conditions.

Fig. 4 is a photograph showing the result of SDS-PAGE (5% gel) for analyzing the solubilized sample of fraction 1 (F1) paste under non-reducing conditions.

Fig. 5 is a photograph showing the result of analyzing vWF-cleaving protease fractions after being subjected to gel filtration chromatography three times using the solubilized sample of F1 paste as a starting material. Fig. 5A is a chart showing gel filtration chromatography, Fig. 5B shows the result of SDS-PAGE on fractions under non-reducing conditions, and Fig. 5C shows the results of SDS-PAGE on vWF-cleaving activity under reducing conditions.

Fig. 6 is a photograph showing the results of analyzing vWF-cleaving protease fractions in which the fraction collected by gel filtration chromatography is purified by DEAE anion exchange chromatography. Fig. 6A is a chart showing gel filtration chromatography, Fig. 6B shows the result of SDS-PAGE (8% gel) on elution fractions under non-reducing conditions, and Fig. 6C shows the results of SDS-PAGE on vWF-cleaving activity under reducing conditions. In Fig. 6C, three bands indicate an intact vWF molecule (remaining uncleaved), a vWF cleavage fragment, and a vWF cleavage fragment, respectively, as in Fig. 5C.

Fig. 7 is a photograph showing an electrophoresed fragment obtained when the vWF-cleaving protease fraction purified and concentrated by DEAE anion exchange chromatography is further purified by Biophoresis-based SDS-PAGE (non-reducing conditions).

Fig. 8 is a photograph showing the result of electrophoresis on a fraction obtained by further purifying a vWF-cleaving protease fraction by Biophoresis-based SDS-PAGE for analyzing vWF-cleaving protease activity and SDS-PAGE on active fractions under reducing conditions. Fig. 8A shows the results of SDS-PAGE for analyzing vWF-cleaving protease activity under non-reducing conditions, and Fig. 8B shows the results of SDS-PAGE for analyzing active fractions under reducing conditions.

Fig. 9 relates to the identification of the vWF-cleaving protease gene, which is a diagram showing primers used for amplifying the gene fragment for a Northern blot probe.

Fig. 10 relates to the identification of the vWF-cleaving protease gene, which is a photograph showing Northern blot autoradiography. Fig. 10A shows the results obtained when the protease-encoding gene is used as a probe, and Fig. 10B shows the results obtained when a β-actin probe (RNA control) is used.

Fig. 11 relates to the identification of the vWF-cleaving protease gene, and is a diagram showing the locations and the sequences of the primers used in the RACE experiments.

Fig. 12 is a diagram showing the locations of primers designed for cloning full-length cDNA.

Fig. 13 is a diagram showing a process for constructing a vector containing full-length cDNA.

Fig. 14 is a photograph showing the expression in various cell lines (Western blotting under reducing conditions using anti-FLAG antibody, where the mock is prepared by inversely inserting a gene in an expression vector). In Fig. 14, each lane shows the results using the indicated sample.

Lane 1: Mock (host: 293 cell)
Lane 2: vWF-cleaving protease, cDNA+FLAG (host: 293 cell)
Lane 3: Mock (host: HepG2 cell)
Lane 4: vWF-cleaving protease, cDNA+FLAG (host: HepG2 cell)
Lane 5: Mock (host: Hela cell)
Lane 6: vWF-cleaving protease, cDNA+FLAG (host: Hela cell)

Fig. 15 is a photograph showing the activity assay of recombinant expression protease (analysis of vWF-cleavage by SDS-PAGE under non-reducing conditions, where the mock is prepared by inversely inserting a gene in an expression vector). In Fig. 15, each lane shows the results using the indicated sample.

Lane 1: Mock (host: Hela cell)
Lane 2: Supernatant in which vWF-cleaving protease was expressed (host: Hela cell)
Lane 3: Mock (host: HepG2 cell)
Lane 4: Supernatant in which vWF-cleaving protease was expressed (host: HepG2 cell)
Lane 5: Mock (host: 293 cell)
Lane 6: Supernatant in which vWF-cleaving protease was expressed (host: 293 cell)
Lane 7: Mock (host: BHK cell)
Lane 8: Supernatant in which vWF-cleaving protease was expressed (host: BHK cell)
Lane 9: Mock (host: COS cell)
Lane 10: Supernatant in which vWF-cleaving protease was expressed (host: COS cell)
Lane 11: Mock (host: CHO cell)
Lane 12: Supernatant in which vWF-cleaving protease was expressed (host: CHO cell)

Fig. 16 is a photograph showing the result of Western blotting using an antibody established against the protease of the present invention, wherein Western blotting is carried out for various antiserums using the 293 cell as a host and a recombinant vWF-cleaving protease. In Fig. 16, each lane shows the results obtained with the use of the indicated sample.

Lane 1: Mouse antiserum (prepared by administering purified protein)
Lane 2: Rabbit antiserum (prepared by hypodermically administering an expression vector to a rabbit)
Lane 3: Untreated rabbit antiserum
Lane 4: Rabbit antiserum (prepared by administering KLH-conjugated partial synthetic peptide)

Fig. 17 is a photograph showing the result of Western blotting using an antibody established against the protease of the present invention, wherein various samples derived from human plasma and recombinant expression units are detected using rabbit antiserum obtained by administering full-length cDNA, of vWF-cleaving protease. In Fig. 17, each lane shows the results obtained with the use of the indicated sample.

Lane 1: Partially purified sample derived from human plasma cryoprecipitate
Lane 2: Purified vWF-cleaving protease derived from human plasma
Lane 3: Gel-filtrated FI paste sample obtained from pooled human plasma
Lane 4: Recombinant vWF-cleaving protease (host: 293 cell)
Lane 5: Recombinant vWF-cleaving protease (host: Hela cell)

Fig. 18 is a photograph showing the result of Western blotting using an antibody established against the protease of the present invention, wherein rabbit antiserum obtained by immunizing a rabbit with a partially synthesized peptide of the vWF-cleaving protease is used to confirm the vWF-cleaving protease in healthy human plasma and that in the plasma and gene recombinant vWF-cleaving protease of a TTP patient. In Fig. 18, each lane shows the results obtained with the use of the indicated sample.

Lane 1: Gel-filtrated FI paste sample obtained from pooled human plasma
Lane 2: Normal human plasma 1
Lane 3: Normal human plasma 2
Lane 4: Normal human plasma 3
Lane 5: TTP patient's plasma 1
Lane 6: TTP patient's plasma 2
Lane 7: Recombinant vWF-cleaving protease (host: 293 cell)

Lane 8: Recombinant vWF-cleaving protease (host: Hela cell)

Fig. 19 is a diagram showing the result of ELISA using an antibody prepared against the vWF-cleaving protease.
Fig. 20 is a photograph showing the result of SDS-PAGE (silver staining) analyzing each fraction of affinity purified vWF-cleaving protease using an antibody under reducing conditions. In Fig. 20, each lane shows the results obtained with the use of the indicated sample.

Lane 1: Applied culture supernatant (diluted 10-fold)
Lane 2: Passed-through fraction
Lane 3: Washed fraction
Lane 4: Elution fraction

Fig. 21 is a photograph showing the results of evaluating neutralizing activity using an antibody (SDS-PAGE for analyzing vWF-cleaving activity under non-reducing conditions). In Fig. 21, each lane shows the results obtained with the use of the indicated sample.

Lane 1: vWF-cleaving protease solution: normal rabbit serum = 1:1
Lane 2: vWF-cleaving protease solution: normal rabbit serum (diluted 5-fold) = 1:1
Lane 3: vWF-cleaving protease solution: peptide-immunized rabbit serum = 1:1
Lane 4: vWF-cleaving protease solution: peptide-immunized rabbit serum (diluted 5-fold) = 1:1
Lane 5: vWF-cleaving protease solution : recombinant protein-immunized rabbit serum = 1:1
Lane 6: vWF-cleaving protease solution : recombinant protein-immunized rabbit serum (diluted 5-fold) = 1:1
Lane 7: vWF-cleaving protease solution: 10mM EDTA = 1:1
Lane 8: vWF-cleaving protease solution: buffer only = 1:1
Lane 9: buffer (without vWF-cleaving protease) : buffer = 1:1

Fig. 22 is a diagram showing the construction of an expression vector for a molecular species lacking a C-terminal domain.

Best Modes for Carrying out the Invention

[0071] The present invention is hereafter described in detail with reference to the following examples, although it is not limited to these examples.

Example 1

(Preparation of vWF)

[0072] A plasma cryoprecipitation (2 g) was dissolved in 20 ml of buffer (0.01 % Tween-80/50 mM Tris-HCl/100 mM NaCl, pH 7.4), and the resultant was subjected to gel filtration using a Sephacryl S-500 HR Column (2.6 x 90 cm, Amersham Pharmacia) to prepare vWF. Fractions were recovered at a flow rate of 2 ml/min in amounts of 6 ml each. vWF was analyzed by Western blotting using a peroxidase-labeled rabbit anti-human vWF antibody (DAKO), and high-molecular-weight vWF fractions were pooled. The pooled fractions were subjected to multimer analysis using agarose electrophoresis as described below.

[0073] As shown in Fig. 1, vWF originally has a multimer structure in which vWF monomer molecules are polymerized with each other at their N-terminuses or at their C-terminuses, and vWF is subjected to partial hydrolysis by the vWF-specific cleaving protease. As a result of the analysis, as shown in Fig. 2, the purified vWF exhibited a multimer pattern based on agarose electrophoresis approximately equivalent to that in the plasma of a healthy person (the ladder in the drawing shows the electrophoresis pattern of vWF having a multimer structure, and the upper portion indicates vWF with advanced polymerization). This can prepare vWF comprising substantially no impurities that degrade it, and this fraction was used as a substrate when assaying the vWF-cleaving activity as described below.

Example 2

(vWF-cleaving reaction)

[0074] vWF-cleaving activity was assayed as follows. A sample comprising 10 mM barium chloride (final concentration) was pre-incubated at 37°C for 5 minutes to activate protease. A buffer (15 to 20 ml, 1.5 M urea/5 mM Tris-HCl,

pH 8.0) was placed in a 50 ml Falcon Tube. Subsequently, a membrane filter (0.025 μm, Millipore) was floated therein, and 100 μl of activated sample prepared by mixing with 50 μl of vWF substrate solution was added. The resultant was allowed to stand in an incubator (37°C) overnight and recovered from the filter on the next day. The recovered sample was evaluated based on the vWF cleavage pattern as described below in the "SDS-PAGE" section.

SDS-PAGE

[0075] SDS-5% polyacrylamide gel was autologously prepared and used. An SDS electrophoresis buffer (2 μl, in the presence or absence of a reducing agent, i.e., 2-mercaptoethanol) was added to 10 μl of the sample described in the "vWF-cleaving activity assay" section, and the resultant was boiled for 3 minutes to prepare an electrophoresis sample. The gel was subjected to electrophoresis at 30 mA for 1 hour and then stained with the Gel Code Blue Stain Reagent (PIERCE) utilizing CBB staining. As shown in Fig. 1, activity is evaluated based on the development of a cleavage fragment and the presence or absence of fragments remaining uncleaved under reducing or non-reducing conditions. This is more specifically described in Example 3 and Fig. 3 below.

Multimer analysis utilizing agarose electrophoresis

Preparation of gel, electrophoresis

[0076] Low gelling temperature agarose (Type VII, Sigma) was added to 375 mM Tris-HCl (pH 6.8) until a concentration of 1.4% was reached, followed by heating in a microwave oven to completely dissolve the gel. Thereafter, 0.1% SDS was added, and the resultant was maintained at 56°C. The resultant was made to flow into a gel mold and solidified by cooling at 4°C overnight (running gel). The next day, high gelling temperate agarose (SeaKem) was mixed with 375 mM Tris-HCl (pH 6.8) until a concentration of 0.8% was reached, and dissolved by boiling in a microwave oven. Thereafter, the resultant was maintained at 56°C (stacking gel). The gel prepared on the previous day was cleaved, leaving a 10-cm fraction from the end uncleaved. The aforementioned gel was made to flow into the cleaved portion, and the gel was made to keep flowing at 4°C for at least 3 hours, followed by solidification. Pyronin Y was added to the sample described in the "vWF cleaving activity assay" section above, and the gel was prepared under non-reducing conditions without boiling. The gel was subjected to electrophoresis at 10 mA for at least 24 hours using an SDS-PAGE buffer.

Western blotting

[0077] After the electrophoresis, the gel was immersed in a transcription buffer (0.005% SDS, 50 mM phosphate buffer, pH 7.4) for 10 minutes, and the resultant was transferred to a nitrocellulose membrane using a transcription apparatus at 4°C at 0.5 A overnight. Blocking was performed using a blotting solution (5% skim milk, PBS) for 30 minutes, and the gel was then allowed to react for at least 6 hours with the peroxidase-labeled rabbit anti-human vWF antibody (DAKO), which was diluted 1,000-fold with the blotting solution. Thereafter, the gel was washed three times with the blotting solution and once with PBS, and color was developed using Konica Immunostain HRP-1000 (Konica), which was a substrate reaction solution for peroxidase. The purified vWF analyzed in this assay was found to have been undegraded, but was sufficiently usable as a substrate in the present invention (Fig. 2).

Example 3

(Preparation of vWF-cleaving protease)

[0078] Plasma was subjected to ethanol fractionation developed by Cohn. A protease having high vWF-cleaving activity (one with high specific activity) when protein levels in four fractions (i.e., starting plasma, cryoprecipitate, fraction I (FI) supernatant, and a paste) are made equivalent to each other was selected. As shown in Fig. 3, the protease activity was highest in the FI paste. The N-terminal sequence of this cleavage fragment was analyzed, and as a result, activity derived from the cryoprecipitate and the FI paste were found to cleave the peptide bond between residues Tyr 842 and Met 843. Thus, the FI paste was determined to be a main starting material for purification thereafter.

Solubilization of FI paste

[0079] The FI paste was fractionated in fractions of 12 g each and then cryopreserved. The paste was allowed to melt at 4°C the day before its use. The next day, 120 ml of solubilizing buffer (0.05% azide, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl) was added at 10 mg/ml, and the mixture was stirred at 37°C for 2 hours. The product was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was then recovered, followed by filtration with a prefilter, a 5.0 μm

filter, and a 0.8 μm filter in that order. The resultant was determined to be a solubilized sample. Fig. 4 shows the result of SDS-PAGE of the solubilized sample.

Gel filtration chromatography of vWF-cleaving protease

[0080] The solubilized F1 paste was applied to a Sephacryl S-300 HR Column (5 x 90 cm, Amersham Pharmacia) to conduct the first gel filtration. A buffer comprising 0.05 % azide, 50 mM Tris-HCl (pH 7.4), and 100 mM NaCl (hereinafter referred to as an "elution buffer"), which was the same as the solubilizing buffer, was used. The flow rate was 5 ml/min, fractionation was initiated at 600 ml after the sample application, and fractions were recovered in amounts of 10 ml each. Fractions were subjected to the vWF-cleaving reaction, and their activities were then analyzed by SDS-PAGE. Fractions that exhibited protease activity were pooled, and a small amount of saturated ammonium sulfate was gradually added dropwide thereto until a final concentration of 33% saturation was reached. The mixture was further allowed to stand at 4°C overnight. The next day, the product was centrifuged at 10,000 rpm for 10 minutes, and an active fraction of interest was recovered as a precipitate. The procedures comprising solubilization, gel filtration, and ammonium sulfate precipitation were performed for 5 batches and the resultant was cryopreserved at -20°C.

[0081] The ammonium sulfate precipitates (2 to 3 batches) obtained by the first gel filtration were dissolved in 50 ml of elution buffer, and passed through the Sephacryl S-300 HR Column (5 x 90 cm) in the same manner as in the first gel filtration to perform the second gel filtration. The elution buffer, conditions, operations, and the like were the same as those in the first gel filtration. Fractions were subjected to the vWF-cleaving reaction, and their activities were then analyzed by SDS-PAGE. Fractions with activity were pooled, and ammonium sulfate precipitation was similarly performed. These procedures were repeated two times.

[0082] The ammonium sulfate precipitates (2 batches) obtained by the second gel filtration were dissolved in 50 ml of elution buffer, and applied to the Sephacryl S-300 HR Column (5 x 90 cm) in the same manner as in the first and the second gel filtration to perform the third gel filtration. The elution buffer, conditions, operations, and the like were the same as those in the first and the second gel filtration. Fractions were subjected to the vWF-cleaving reaction, and their activities were then analyzed by SDS-PAGE, followed by pooling. Fig. 5 shows SDS-PAGE for analyzing these fractions and that for analyzing vWF-cleaving activity. Based on the patterns of gel filtration and the data showing activity, the protease of the present invention was found to be eluted in the region between fraction 37 and fraction 47. Based on a separately conducted elution experiment for high-molecular-weight gel filtration marker (Amersham Pharmacia), this site of elution was deduced to have a molecular weight equivalent to 150 to 300 kDa. In this phase, considerable amounts of impurities were still present.

DEAE anion exchange chromatography

[0083] The pooled fraction obtained by three gel filtration operations was subjected to dialysis overnight with a buffer comprising 50 mM Tris-HCl and 50 mM NaCl (pH 7.1). After the dialysis, anion exchange chromatography was performed using a 5 ml HiTrap DEAE-Sepharose Fast Flow Column (Pharmacia) to conduct further purification and concentration. Equilibrating and washing were performed using a buffer comprising 50 mM Tris-HCl (pH 7.1), and elution was performed using 0.25 M NaCl. The flow rate was 5 ml/min, and 5 fractions of 5 ml each were recovered and pooled. Fig. 6 shows the results of SDS-PAGE for analyzing elution fractions and those for analyzing vWF-cleaving activity. Based on SDS-PAGE for activity assay, the protease of the present invention having vWF-cleaving activity was considerably effectively concentrated in the elution fraction.

Fractionation utilizing SDS-PAGE

[0084] The sample (5 ml) purified and concentrated by DEAE anion exchange chromatography was further concentrated to 0.5 ml using Centricon (molecular weight cut off: 10,000 Da, Amicon). The protease of the present invention was isolated by Biophoresis III (Atto Corporation) utilizing SDS-PAGE. In accordance with the Laemmli method (Nature, vol. 227, 680-685, 1970), a buffer for electrophoresis tanks was prepared, and developed with 8% polyacrylamide gel to recover the electrophoresis fraction. Fig. 7 shows the result of SDS-PAGE for analyzing the recovered fractions. The buffer used for recovery was comprised of 50 mM Tris-HCl and 10% glycerol (pH 8.8). As is apparent from Fig. 7, this process according to the present invention has a high ability to produce separation. Fig. 8 shows the results of analyzing activity of a fraction further purified by electrophoresis and the results of SDS-PAGE for analyzing active fractions. The protease of the present invention can be recovered as an active molecule even after SDS-PAGE. When the activity of this protease in the plasma is determined to be 1 in terms of specific activity, a degree of purification of 30,000- to 100,000-fold was deduced to be achieved based on the average protein content in the plasma (60 mg/ml).

Example 4

(Partial amino acid sequencing)

[0085] The partial amino acid sequence of the isolated protease was determined. This protease, which was isolated using Biophoresis, was transferred to a PVDF membrane after SDS-PAGE by a conventional technique, air-dried, and then subjected to analysis using the automated protein sequencer (model 492; PE Applied Biosystems). As a result, the vWF-cleaving protease of the present invention isolated under the above conditions was found to comprise a polypeptide chain having a molecular weight of 105 to 160 kDa in SDS-PAGE under reducing conditions. This protease was also found to have, as a partial sequence, Leu-Leu-Val-Ala-Val, and preferably Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val.

Deduction of isolated protease utilizing bioinformatics

[0086] At present, bioinformatics enables the deduction of full nucleotide sequences encoding a polypeptide without substantial gene cloning through collation with information in the database accumulated in the past (BIOINFORMATICS: A Practical Guide to the Analysis of Genes and Proteins, edited by Andreas D. Baxevanis and B. F. Francis Ouellette). Based on the partial amino acid sequencing by the aforementioned process (Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val), the database was searched by the tblastn program. As a result, a chromosome clone (AL158826) that was deduced to encode the protease of the present invention was identified by genomic database search. Further, a part of the protease of interest as the expressed sequence tag (EST) and a clone that was deduced to be a part of the polypeptide encoded by the aforementioned genome (AI346761 and AJ011374) were identified. The amino acid sequence as shown in SEQ ID NO: 3 or 7 was deduced based thereon to be an active vWF-cleaving protease site.

Example 5

(Gene identification)

[0087] Synthesis of all the following synthetic primers was performed by Greiner Japan Co.Ltd. by request. Further, reagents used for gene recombination were those manufactured by TAKARA, TOYOBO, and New England Biolabs unless otherwise specified.

Preparation of a gene fragment as a Northern blotting probe

[0088] A sense primer (SEQ ID NO: 9) and an antisense primer (SEQ ID NO: 10) were prepared. PCR was carried out using Universal QUICK-Clone™ cDNA (Clontech), which was a mixture of cDNA derived from normal human tissue, as a template and TaKaRa LA Taq with GC rich buffer. A gene sandwiched between these primers was amplified, and the amplified fragment was cloned using a TOPO TA cloning™ kit (Invitrogen). DNAs having the nucleotide sequence as shown in SEQ ID NO: 6 were isolated from several clones.

[0089] A vector portion was removed from this cloned DNA by EcoRI digestion, separated and purified by agarose electrophoresis, and the resultant was determined to be a template for preparing probes for Northern blotting.

Northern blotting

[0090] The gene fragment prepared above was employed as a template to prepare a radioactive probe using [α-^{32}P] dCTP (Amersham Pharmacia) and a BcaBEST™ labeling kit (TAKARA). Hybridization was carried out using the Human 12-lane Multiple Tissue Northern Blots™ (Clontech) filter in accordance with the method described in Molecular Cloning 2nd Edition, pp. 9.52-9.55. Detection was carried out by autoradiography. As shown in Fig. 10, mRNA encoding the protease was expressed mainly in the liver. The size of this mRNA was found to be more than 4.4 kb.

Isolation and identification of gene encoding the protease

[0091] As a result of Northern blotting, mRNA was found to be expressed mainly in the liver. Thus, the protease gene of the present invention was isolated and identified in accordance with the RACE technique using normal human liver-derived poly A$^+$ RNA and Marathon-Ready™ cDNA (Clontech).

[0092] More specifically, the first PCR was carried out as 5' RACE using normal human liver-derived Marathon-Ready™ cDNA in accordance with the product's manual and using the AP-1 primer attached to the kit and antisense

primers (SEQ ID NOs: 11 to 13) arbitrarily selected from the group of Gene Specific Primers (GSP) excluding the primer 1 located in the uppermost stream as shown in Fig. 11. Nested PCR (the second PCR) was then carried out using the AP-2 primer located in the inside thereof and the antisense primer located in the inside of the primer used for the first PCR as shown in Fig. 11. Thereafter, TA cloning was earned out. Genes were prepared from the developed colonies in accordance with a conventional technique (Molecular Cloning 2nd Edition, pp. 1.25-1.28), and nucleic acid sequences were decoded using an automatic DNA sequencer. The primer used for sequencing was the primer used for PCR or a primer located in the inside thereof. Further, the primer was designed based on the sequence determined after serial decoding.

[0093] 3' RACE was started from normal human liver-derived poly A^+ RNA using the 3'-Full RACE Core Set (TAKARA), and reverse transcription was carried out in accordance with the attached manual using the attached oligo dT primer. The band amplified by PCR using the sense primer (SEQ ID NO:14) located at "primer 2" in Fig. 11 and the attached oligo dT primer was separated by agarose electrophoresis and extracted, followed by TA cloning. Genes were prepared from the developed colonies, and nucleic acid sequences were decoded using an automatic DNA sequencer. A primer used for sequencing was designed based on the sequence determined after serial decoding.

Example 6

(Preparation of a vector comprising full-length cDNA 1)

[0094] cDNA encoding the protein was subjected to one-stage PCR by, for example, using a sense primer 1 (SEQ ID NO: 22) comprising an XhoI restriction site and an initiation codon and an antisense primer 2 (SEQ ID NO: 23) comprising an SalI restriction site and a termination codon (see Fig. 12), using the aforementioned normal human liver-derived Marathon-Ready™ cDNA as a template and the TaKaRa LA Taq with GC rich buffer, followed by the aforementioned TA cloning. Thereafter, the full length of the product was confirmed using an automatic DNA sequencer.

Example 7

(Preparation of a vector comprising full-length cDNA 2)

[0095] Restriction sites AccI and AvrII that cleaved cDNA only at one point on the inner sequence of the cDNA (SEQ ID NO: 15) encoding the protein were found. With the use thereof, full-length cDNA was divided into three fragments as shown in Fig. 12. A fragment 1 sandwiched between the sense primer 1 (SEQ ID NO: 22) and the antisense primer 3 (SEQ ID NO: 24), a fragment 2 sandwiched between the sense primer 4 (SEQ ID NO: 25) and the antisense primer 5 (SEQ ID NO: 26), and a fragment 3 sandwiched between the sense primer 6 (SEQ ID NO: 27) and the antisense primer 2 (SEQ ID NO: 23) were provided, respectively, in each of the above three fragments. Each fragment was subjected to PCR using the aforementioned normal human liver-derived Marathon-Ready™ cDNA as a template and TaKaRa LA Taq with GC rich buffer, followed by the aforementioned TA cloning. The full length of the product was confirmed using an automatic DNA sequencer. Further, the pCR 2.1 vector included in the aforementioned TA cloning kit was subjected to self ligation, the ligation product was cleaved with XhoI/HindIII, ligated to a linker comprising XhoI/AccI/AvrII/HindIII (prepared by annealing the synthetic DNA as shown in SEQ ID NO: 28 or 29), and the three aforementioned fragments were sequentially ligated in a conventional manner to bind them. Thus, cDNA comprising the entire region was prepared (see Fig. 13).

Example 8

(Preparation of an expression vector comprising full-length cDNA: an animal cell host)

[0096] DNA obtained in Example 6 or 7 was digested with restriction enzymes XhoI/SalI, ligated to, for example the SalI site in the pCAG vector (Niwa, H. et al., Gene, vol. 108, 193-199), and the direction of the insertion and the full-length sequence were confirmed using an automatic DNA sequencer.

Example 9

(Transfection of an expression vector comprising full-length cDNA into an animal cell)

[0097] The animal cell expression vector prepared in Example 8 was transfected in the following manner using the 293 cell (human embryonic kidney cell line), the Hela cell, and the HepG2 cell. At the outset, cells were disseminated at 1 to 3 x 10^5 cells per 35 mm dish 24 hours before the transfection. The next day, 2 μl of polyamine transfection

reagent, TransIT (TAKARA), per μg of the expression vector, were added to 100 μl of a serum-free medium such as Opti-MEM to prepare a complex with DNA in accordance with the instructions included with the reagent. Thereafter, the complex was added dropwise to the various types of previously prepared cells, and the resultants were incubated for 2 to 8 hours, followed by medium exchange. The medium was further exchanged three days later with the selective medium to which G418 had been added. Thereafter, medium was exchanged every three days to produce a stably expressed strain. An example thereof is shown in Fig. 14 as a temporarily expressed strain comprising an FLAG epitope tag at its C-terminus. Detection was carried out by Western blotting using the anti-FLAG-M2 antibody (Kodack) and staining with anti-mouse Ig-alkaline phosphatase-labeled antibody system. The recombinant strain expressed using cDNA as shown in this example exhibited a molecular size of about 250 kDa under reducing conditions. This molecular size was also found in the plasma of a healthy human (Fig. 18, Example 14 below). Several different molecular species of this protease are found to be present in the human plasma, which could be caused by the presence of the alternative splicing products (SEQ ID NOs: 6 to 21) observed at the time of gene cloning, difference in post-translational modification such as sugar chain addition, or degradation during purification (described in Example 14 and in Fig. 17 of the present invention and Gerritsen et al., Blood, vol. 98, 1654-1661 (2001)).

[0098] Subsequently, the vWF-cleaving activity of the recombinant strain was confirmed by the method described in Example 2 (Fig. 15). As a result, the human plasma-derived protease and the gene recombinant product of the present invention were found to exhibit the same vWF-cleaving activities.

Example 10

(Preparation of an expression vector comprising partial cDNA: an *E. coli* host)

[0099] Partial cDNA encoding the metalloprotease domain of the protein was subjected to PCR using a sense primer comprising an NcoI restriction site and an initiation codon (SEQ ID NO: 30). and an antisense primer comprising an HindIII restriction site and a termination codon (SEQ ID NO: 31), the aforementioned normal human liver-derived Marathon-Ready™ cDNA or the cDNA obtained in Example 6 or 7 as a template, and the TaKaRa LA Taq with GC rich buffer. The PCR product was then digested with NcoI/HindIII, ligated to the NcoI/HindIII digest of an *E. coli* expression vector such as pUT1 (Soejima et al., J. Biochem. Tokyo, vol. 130, 269-277 (2001)), and transformed to the *E. coli* competent cell JM 109 by a conventional technique. Several clones were collected from the formed colony group, and genes were prepared therefrom. Thereafter, the resulting genes were confirmed to be the genes encoding the polypeptide, wherein the nucleic acid sequence of the insertion site of the plasmid vector was equivalent to SEQ ID NO: 32 or substantially represented by SEQ ID NO: 33, using an automatic DNA sequencer.

Example 11

(Expression of partial cDNA-containing expression vector in *E. coli*)

[0100] An *E. coli* host with the expression vector constructed in Example 10 introduced therein was precultured in 200 ml of LB medium comprising 50 μg/ml ampicillin at 30°C overnight. The resultant was sowed in a fermenter comprising 8 liters of LB medium, and culture was conducted at 30°C until the turbidity at 600 nm became 0.2 to 0.5. Thereafter, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the mixture was further cultured overnight to induce the metalloprotease domain of the protein to be expressed. The cultured *E. coli* were collected using a centrifuge (4°C for 30 minutes).

[0101] Subsequently, the collected *E. coli* pellet was resuspended in distilled water, and lysozyme (final concentration: 0.6 mg/ml) was added thereto. The mixture was stirred at room temperature for 30 minutes, allowed to stand at 4°C overnight, and cells were then destroyed. After the ultrasonication, centrifugation was carried out using a centrifuge (4°C for 20 minutes), and the pellet was recovered. The recovered pellet was resuspended in a buffer comprising 50 mM Tris, 10 mM EDTA, and 1% Triton X-100 (pH 8.0). These procedures of centrifugation, ultrasonication, and resuspension were repeated several times, and the pellet was then resuspended in distilled water. Similarly, procedures of centrifugation, ultrasonication, and resuspension were repeated several times to recover an inclusion body. This inclusion body was used as an antigen when producing an antibody.

Example 12

(Isolation of homologous gene of other animal species)

[0102] The nucleic acid sequence as shown in SEQ ID NO: 15 was used as a probe, and a homology search was conducted using the BLASTN program at the GenomeNet WWW server (http://www.genome.ad.jp/). As a result, chro-

mosome clones AC091762 and AC090008 that were mapped at mouse chromosome 10 were obtained. Based on these sequences, a mouse homolog of the protease of the present invention as shown in SEQ ID NO: 34 was deduced. A new primer was designed from this sequence, and Northern blot analysis was conducted by the technique used in isolating and identifying the gene encoding the human vWF-cleaving protease. Thus, the occurrence of the specific expression in the liver was observed as with the case of humans. Further, normal mouse liver-derived poly A+ RNA and Marathon-Ready™ cDNA (Clontech) were used to isolate and identify the protease gene of the present invention by the RACE technique as in the case of humans. As a result, the mouse homologous gene sequences of the protease as shown in SEQ ID NOs: 35 and 36 were determined.

[0103] Based on the thus determined mouse homologous partial sequence, the Exon/Intron structure on the 5' side of the aforementioned mouse chromosome 10 was determined. In accordance with a conventional technique (e.g., Gene Targeting: A Practial Approach First Edition, edited by A. L. Joyner, Teratocarcinomas and embryonic stem cell a practical approach), a targeting vector for knock-out (knock-in) mice can be prepared based thereon. This enabled the production of mutated mice. Further, this protein can be subjected to recombinant expression by a conventional technique.

Example 13

(Production of an antibody and construction of a detection system for the present protease using the antibody)

[0104] In accordance with a conventional technique (e.g., Current Protocols in Molecular Biology: Chapter 11 immunology, Antibody Engineering: A PRACTICAL APPROACH, edited by J. McCAFFERTY et al. or ANTIBODY ENGINEERING second edition, edited by Carl A. K. BORREBAECK), an expression vector was administered to a mouse or rat. This expression vector comprises a substance prepared by optionally binding an antigen protein partially purified from human plasma or a synthetic peptide having a partial amino acid sequence thereof (e.g., a C-terminal peptide sequence (SEQ ID NO: 37) Phe-Ser-Pro-Ala-Pro-Gln-Pro-Arg-Arg-Leu-Leu-Pro-Gly-Pro-Gln-Glu-Asn-Ser-Val-Gln-Ser-Ser, which was one isoform of the protease of the present invention) to an optimal carrier substance such as KLH (Cys was added to, for example, the N- or C-terminus to facilitate KLH addition), the aforementioned gene recombinant protein, or a gene encoding this protein. Thus, a monoclonal antibody-expressing hybridoma was established, and a polyclonal antibody (antiserum) was produced.

[0105] Subsequently; the antibodies prepared by the various aforementioned techniques were used to detect the protease of the present invention by Western blotting in accordance with a conventional technique (e.g., Current Protocols in Molecular Biology: Chapter 10 analysis of proteins, Chapter 11 immunology). More specifically, the culture supernatant of the recombinant unit-expressing 293 cell obtained in the procedure as described in Example 9 was subjected to SDS-PAGE under non-reducing conditions, transferred to a PVDF membrane, and confirmed using mouse or rabbit antiserum to confirm the expression of the genetically recombinant unit (Fig. 16). As a result, a band that was deduced to be derived from the protease of the present invention was found in a molecular size range of 160 to 250 kDa. Subsequently, the protease of the present invention was detected using starting plasma or the like and a recombinant unit under non-reducing conditions. As a result, a band was found in 105 to 160 kDa or 160 to 250 kDa (Fig. 17). Also, a band derived from a similar recombinant unit was detected in a monoclonal antibody established by immunizing a recombinant protein (clone No. CPHSWH-10).

[0106] Further, the C-terminal peptide sequence Phe-Ser-Pro-Ala-Pro-Gln-Pro-Arg-Arg-Leu-Leu-Pro-Gly-Pro-Gln-Glu-Asn-Ser-Val-Gln-Ser-Ser (SEQ ID NO: 37), which was one isoform of the protease of the present invention, was bound to KLH. The resultant was used as an immunogen to obtain a peptide antibody. With the use thereof, the protease of the present invention was detected from the plasma of healthy persons, plasma of TTP patients, or a culture supernatant of the recombinant unit under reducing conditions. As a result, a band of approximately 250 kDa that was deduced to be a signal derived from the protease of the present invention was found, although it was not clear based on plasma derived from some TTP patients (Fig. 18).

[0107] Furthermore, enzyme immunoassay (ELISA) constructed by combining the obtained antibodies enabled the preparation of a calibration curve that is concentration-dependent at the culture supernatant level of the recombinant protein (Fig. 19). An example of ELISA is as follows. The obtained mouse anti-vWF-cleaving protease antibody was immobilized on the Maxisorp plate (Nunc), and 1/1, 1/2, and 1/4 diluents of the culture supernatant of the vWF-cleaving protease-temporarily expressing 293 cells were allowed to react in amounts of 100 μl/well (Mock supernatant as "0"). The plate was subjected to reaction, for example, at 37°C for 1 hour, and then washed with 0.05% Tween 20/TBS. Thereafter, the 100-fold diluted rabbit anti-vWF-cleaving protease antibody was allowed to react in amounts of 100 μl/well, for example, at 37°C for 1 hour, and the plate was washed with 0.05% Tween 20/TBS. The 1,000-fold diluted peroxidase-labeled anti-rabbit Ig antibody (BioRad) was then allowed to react in amounts of 100 μl/well, for example, at 37°C for 1 hour, and the plate was washed with 0.05% Tween 20/TBS. Thereafter, color was developed for a given period of time using a coloring substrate TMBZ, the reaction was terminated using 1M sulfuric acid as a termination

liquid, and the absorbance at 450 nm was assayed. The application thereof enabled the quantification of the protease of the present invention in a variety of specimens.

Example 14

(Purification of the protease using an antibody)

[0108] The obtained antibody was bound to a suitable immobilization carrier to prepare an affinity column, and the resulting column was used to purify. the protease of the present invention. The affinity column was prepared by immobilizing an antibody using Cellulofine for NHS activation (Chisso Corporation) in accordance with the included instructions. The thus prepared swollen carrier (about 1 ml) was used to apply the culture supernatant in which the recombinant gene had been expressed in the 293 cell of the protease as described in Example 9. Thereafter, the column was washed with 50 mM Tris-HCl and 0.1M NaCl (pH 7.5, hereafter referred to as "TBS"), and elution was carried out using a urea-containing 0.1M glycine buffer (pH 3). The eluted fraction was neutralized with 1M Tris-HCl (pH 8.5) and then dialyzed against TBS. Fig. 20 shows the results of SDS-PAGE analysis of the resulting purified protease. Also, the resulting purified fraction was found to have vWF-cleaving activity. The cleavage point of the vWF fragmented by this recombinant protease was found to be the position between residues Tyr 842 and Met 843 based on the analysis of the N-terminal amino acid sequence of the fragment. Also established were clones (e.g., Clone Nos. CPHSWH-7.2 and 10) that could be similarly subjected to purification with the use of the monoclonal antibody prepared by the method as described in Example 13.

[0109] Subsequently, the partial amino acid sequence of the purified protease was determined. In accordance with a conventional technique, the protease was subjected to SDS-PAGE, transferred to a PVDF membrane, air-dried, and then subjected to analysis using an automated protein sequencer (model 492; PE Applied Biosystems). As a result, the protease was found to comprise Ala-Ala-Gly-Gly-Ile- as a partial N-terminal sequence. This sequence was congruous with the N-terminal sequence of the mature unit of the protease of the present invention, that was deduced from the genetic construction.

Example 15

(Neutralization of the protease activity using an antibody)

[0110] Activity of the aforementioned rabbit polyclonal antibody to neutralize the vWF-cleaving protease was evaluated. Normal rabbit serum, rabbit antiserum comprising the C-terminal peptide sequence (SEQ ID NO: 37), Phe-Ser-Pro-Ala-Pro-Gln-Pro-Arg-Arg-Leu-Leu-Pro-Gly-Pro-Gln-Glu-Asn-Ser-Val-Gln-Ser-Ser bound to KLH as an immunogen, and antiserum, the immunity of which had been induced by the protein expressed by the expression vector as shown in Example 7 or 8, were respectively allowed to pre-react at 37°C for 1 hour with 1 to 10 μg/ml of gene recombinant vWF-cleaving protease (approximated by the Bradford technique) at a volume ratio of 1:1. Alternatively, a 5-fold diluted antiserum was allowed to pre-react under the above conditions with the protease at a volume ratio of 1:1. Thereafter, vWF-cleaving activity was evaluated by the method described above. As a result, it was found that antiserum, which had activity of inhibiting the protease of the present invention, were prepared by immunizing the protein (Fig.21). (antagonist activity) (a metalloprotease inhibitor, i.e., EDTA, was determined to be a control). This indicates the possibility of constructing an acquired TTP patient-like model having a positive autoantibody against vWF-cleaving protease as well as the simple possibility of producing a neutralizing antibody.

Example 16

(Construction of C-terminus deleted modification unit)

[0111] Based on the strategy shown in Fig. 22, the full-length vWF-cleaving protease gene cloning vector (pCR 2.1 vWFCP) obtained in Example 6 or 7 was used to add a variant lacking domains located in a position following the C-terminus (T1135stop, W1016stop, W897stop, T581stop, and Q449stop: each numerical value indicates the number of amino acid residues between Met encoded by the initiation codon AGT and the termination codon, and indicates a site comprising the FLAG epitope (DNA sequence: gactacaaggacgatgacgataagtga (SEQ ID NO: 47) and amino acid sequence: Asp Tyr Lys Asp Asp Asp Asp Lys (SEQ ID NO: 48)). Primers used herein are as follows. "S" indicates a sense primer, and "AS" indicates an antisense primer. Genes Stu I-S (SEQ ID NO: 38), Acc I-S (SEQ ID NO: 39), Avr II-S (SEQ ID NO: 40), Q449stop-AS (SEQ ID NO: 41), T581stop-AS (SEQ ID NO: 42), W897stop-AS (SEQ ID NO: 43), W1016stop-AS (SEQ ID NO: 44), T1135stop-AS (SEQ ID NO: 45), and full-length-AS (SEQ ID NO: 46) were prepared and incorporated in the pCAG expression vector in accordance with the method as used in Examples 8 and

9. This expression vector was introduced in the Hela cell. The primer pair shown at the bottom of the restriction map in the upper portion of Fig. 22 was used to obtain PCR fragments (A) to (F). Each PCR fragment was ligated to pCR 2.1 vWFCP. Further, the resultant was digested with StuI/SalI, and fragments (A) and (B) were digested with StuI/SalI and then ligated. These fragments were further digested with AccI, and fragment (C) was also digested with AccI, followed by ligation. The ligation product was digested with AvrII/SalI, and fragments (D), (E), and (F) were also digested with AvrII/SalI, followed by ligation. As a result, a variant lacking a region between the C-terminus and the position W897 was found to have activity, although it was the result of qualitative analysis. Such a way of approach enables the identification of various functional domains. The design of molecules comprising these domains and having no protease activity is considered to realize the design of antagonists or agonists.

Industrial Applicability

[0112] The findings of the present invention have led to the possibility of replacement therapy for patients having diseases resulting from deficiency of a protease, such as thrombotic thrombocytopenic purpura. This also realizes the establishment of methods for gene cloning and efficient purification from serum or plasma. In particular, the information provided by the present invention enables gene recombination based on the obtained nucleotide sequence and stable production and provision of the protease according to the present invention, which have been heretofore difficult to achieve. Also, these can be applied to replacement therapy for TTP patients, inhibition of platelet plug formation involved with heart infarction or brain infarction, inhibition of arteriosclerosis, prevention of restenosis, reembolization, or infarction involved with PTCA, prevention of reembolization involved with PTCR, and prevention of platelet plug formation caused by HUS or O-157. Diagnosis and therapy utilizing the gene encoding the protease of the present invention or an antibody thereagainst can be realized.

[0113] All publications cited herein are incorporated herein in their entirety. A person skilled in the art would easily understand that various modifications and changes of the present invention are feasible within the technical idea and the scope of the invention as disclosed in the attached claims. The present invention is intended to include such modifications and changes.

SEQUENCE LISTING

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1325 1330 1335

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gaa gga acc 4059

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1 5 10 15

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Pro Asp Val Phe Gln Ala His Gln Glu Asp Thr Glu Arg Tyr Val

20 25 30

ctc acc aac ctc aac atc ggg gca gaa ctg ctt cgg gac ccg tcc 135

Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser

35 40 45

ctg ggg gct cag ttt cgg gtg cac ctg gtg aag atg gtc att ctg 180

Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu

50 55 60

aca gag cct gag ggt gct cca aat atc aca gcc aac ctc acc tcg 225

Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr Ser

65 70 75

tcc ctg ctg agc gtc tgt ggg tgg agc cag acc atc aac cct gag 270
Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro Glu
80 85 90

gac gac acg gat cct ggc cat gct gac ctg gtc ctc tat atc act 315
Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
95 100 105

agg ttt gac ctg gag ttg cct gat ggt aac cgg cag gtg cgg ggc 360
Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
110 115 120

gtc acc cag ctg ggc ggt gcc tgc tcc cca acc tgg agc tgc ctc 405
Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
125 130 135

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Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
140 145 150

gag att ggg cac agc ttc ggc ctg gag cac gac ggc gcg ccc ggc 495
Glu Ile Gly His Ser Phe Gly Leu Glu His Asp Gly Ala Pro Gly
155 160 165

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Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala
170 175 180

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Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln
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Leu Leu Ser Leu Leu Ser Ala Gly Arg Ala Arg Cys Val Trp Asp
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ccg ccg cgg cct caa ccc ggg tcc gcg ggg cac ccg ccg gat gcg 675

Pro Pro Arg Pro Gln Pro Gly Ser Ala Gly His Pro Pro Asp Ala
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Gln Pro Gly Leu Tyr Tyr Ser Ala Asn Glu Gln Cys Arg Val Ala
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Phe Gly Pro Lys Ala Val Ala Cys Thr Phe Ala Arg Glu His Leu
245 250 255

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275 280 285

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Cys Gly Val Glu Lys Trp Cys Ser Lys Gly Arg Cys Arg Ser Leu
290 295 300

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Val Glu Leu Thr Pro Ile Ala Ala Val His Gly Arg Trp Ser Ser
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Trp Gly Pro Arg Ser Pro Cys Ser Arg Ser Cys Gly Gly Gly Val
320 325 330

gtc acc agg agg cgg cag tgc aac aac ccc aga cct gcc ttt ggg 1035
Val Thr Arg Arg Arg Gln Cys Asn Asn Pro Arg Pro Ala Phe Gly
335 340 345

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Gly Arg Ala Cys Val Gly Ala Asp Leu Gln Ala Glu Met Cys Asn

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tgc gcc agg acc gac ggc cag ccg ctg cgc tcc tcc cct ggc ggc      1170
Cys Ala Arg Thr Asp Gly Gln Pro Leu Arg Ser Ser Pro Gly Gly
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gcc tcc ttc tac cac tgg ggt gct gct gta cca cac agc caa ggg      1215
Ala Ser Phe Tyr His Trp Gly Ala Ala Val Pro His Ser Gln Gly
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gat gct ctg tgc aga cac atg tgc cgg gcc att ggc gag agc ttc      1260
Asp Ala Leu Cys Arg His Met Cys Arg Ala Ile Gly Glu Ser Phe
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atc atg aag cgt gga gac agc ttc ctc gat ggg acc cgg tgt atg      1305
Ile Met Lys Arg Gly Asp Ser Phe Leu Asp Gly Thr Arg Cys Met
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cca agt ggc ccc cgg gag gac ggg acc ctg agc ctg tgt gtg tcg      1350
Pro Ser Gly Pro Arg Glu Asp Gly Thr Leu Ser Leu Cys Val Ser
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Gly Ser Cys Arg Thr Phe Gly Cys Asp Gly Arg Met Asp Ser Gln
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cag gta tgg gac agg tgc cag gtg tgt ggt ggg gac aac agc acg      1440
Gln Val Trp Asp Arg Cys Gln Val Cys Gly Gly Asp Asn Ser Thr
                    470                 475                 480
tgc agc cca cgg aag ggc tct ttc aca gct ggc aga gcg aga gaa      1485
Cys Ser Pro Arg Lys Gly Ser Phe Thr Ala Gly Arg Ala Arg Glu
                    485                 490                 495
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Tyr Val Thr Phe Leu Thr Val Thr Pro Asn Leu Thr Ser Val Tyr
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att gcc aac cac agg cct ctc ttc aca cac ttg gcg gtg agg atc 1575
Ile Ala Asn His Arg Pro Leu Phe Thr His Leu Ala Val Arg Ile
515 520 525

gga ggg cgc tat gtc gtg gct ggg aag atg agc atc tcc cct aac 1620
Gly Gly Arg Tyr Val Val Ala Gly Lys Met Ser Ile Ser Pro Asn
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Thr Thr Tyr Pro Ser Leu Leu Glu Asp Gly Arg Val Glu Tyr Arg
545 550 555

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Val Ala Leu Thr Glu Asp Arg Leu Pro Arg Leu Glu Glu Ile Arg
560 565 570

atc tgg gga ccc ctc cag gaa gat gct gac atc cag gtt tac agg 1755
Ile Trp Gly Pro Leu Gln Glu Asp Ala Asp Ile Gln Val Tyr Arg
575 580 585

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Arg Tyr Gly Glu Glu Tyr Gly Asn Leu Thr Arg Pro Asp Ile Thr
590 595 600

ttc acc tac ttc cag cct aag cca cgg cag gcc tgg gtg tgg gcc 1845
Phe Thr Tyr Phe Gln Pro Lys Pro Arg Gln Ala Trp Val Trp Ala
605 610 615

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Ala Val Arg Gly Pro Cys Ser Val Ser Cys Gly Ala Gly Leu Arg
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Glu Thr Val Gln Cys Gln Gly Ser Gln Gln Pro Pro Ala Trp Pro
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gag gcc tgc gtg ctc gaa ccc tgc cct ccc tac tgg gcg gtg gga      2025
Glu Ala Cys Val Leu Glu Pro Cys Pro Pro Tyr Trp Ala Val Gly
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gac ttc ggc cca tgc agc gcc tcc tgt ggg ggc ggc ctg cgg gag      2070
Asp Phe Gly Pro Cys Ser Ala Ser Cys Gly Gly Gly Leu Arg Glu
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cgg cca gtg cgc tgc gtg gag gcc cag ggc agc ctc ctg aag aca      2115
Arg Pro Val Arg Cys Val Glu Ala Gln Gly Ser Leu Leu Lys Thr
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Leu Pro Pro Ala Arg Cys Arg Ala Gly Ala Gln Gln Pro Ala Val
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Ala Leu Glu Thr Cys Asn Pro Gln Pro Cys Pro Ala Arg Trp Glu
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Val Ser Glu Pro Ser Ser Cys Thr Ser Ala Gly Gly Ala Gly Leu
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gct cca gtg act gag ggg cct ggc tcc gta gat gag aag ctg cct      2340
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cat ctg gat gcc acc tct gca ggg gag aag gct ccc tcc cca tgg      2430
His Leu Asp Ala Thr Ser Ala Gly Glu Lys Ala Pro Ser Pro Trp
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ggc agc atc agg acg ggg gct caa gct gca cac gtg tgg acc cct      2475
Gly Ser Ile Arg Thr Gly Ala Gln Ala Ala His Val Trp Thr Pro
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Leu Arg Phe Leu Cys Met Asp Ser Ala Leu Arg Val Pro Val Gln
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gaa gag ctg tgt ggc ctg gca agc aag cct ggg agc cgg cgg gag      2610
Glu Glu Leu Cys Gly Leu Ala Ser Lys Pro Gly Ser Arg Arg Glu
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gtc tgc cag gct gtc ccg tgc cct gct cgg tgg cag tac aag ctg      2655
Val Cys Gln Ala Val Pro Cys Pro Ala Arg Trp Gln Tyr Lys Leu
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gcg gcc tgc agc gtg agc tgt ggg aga ggg gtc gtg cgg agg atc      2700
Ala Ala Cys Ser Val Ser Cys Gly Arg Gly Val Val Arg Arg Ile
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ctg tat tgt gcc cgg gcc cat ggg gag gac gat ggt gag gag atc      2745
Leu Tyr Cys Ala Arg Ala His Gly Glu Asp Asp Gly Glu Glu Ile
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920 925 930

gag gcc tgc agc ctg gag ccc tgc cca cct agg tgg aaa gtc atg 2835
Glu Ala Cys Ser Leu Glu Pro Cys Pro Pro Arg Trp Lys Val Met
935 940 945

tcc ctt ggc cca tgt tcg gcc agc tgt ggc ctt ggc act gct aga 2880
Ser Leu Gly Pro Cys Ser Ala Ser Cys Gly Leu Gly Thr Ala Arg
950 955 960

cgc tcg gtg gcc tgt gtg cag ctc gac caa ggc cag gac gtg gag 2925
Arg Ser Val Ala Cys Val Gln Leu Asp Gln Gly Gln Asp Val Glu
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Val Asp Glu Ala Ala Cys Ala Ala Leu Val Arg Pro Glu Ala Ser
980 985 990

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Val Pro Cys Leu Ile Ala Asp Cys Thr Tyr Arg Trp His Val Gly
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Lys Leu Leu Asp Met Thr Phe Ser Ser Lys Thr Asn Thr Leu Val
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Ser Glu Gly Phe Leu Lys Ala Gln Ala Ser Leu Arg Gly Gln Tyr
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Pro Asp Val Phe Gln Ala His Gln Glu Asp Thr Glu Arg Tyr Val
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Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro Glu
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Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala
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Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln
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290 295 300

ctc ctc gtt cct ctc ctg gat ggg aca gaa tgt ggc gtg gag aag 945
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Lys Thr Gln Leu Glu Phe Met Ser Gln Gln Cys Ala Arg Thr Asp
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His Met Cys Arg Ala Ile Gly Glu Ser Phe Ile Met Lys Arg Gly
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Gly Ser Phe Thr Ala Gly Arg Ala Arg Glu Tyr Val Thr Phe Leu
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530 535 540

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Asp Arg Leu Pro Arg Leu Glu Glu Ile Arg Ile Trp Gly Pro Leu
    590                 595                 600
cag gaa gat gct gac atc cag gtt tac agg cgg tat ggc gag gag      1845
Gln Glu Asp Ala Asp Ile Gln Val Tyr Arg Arg Tyr Gly Glu Glu
605                 610                 615
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Tyr Gly Asn Leu Thr Arg Pro Asp Ile Thr Phe Thr Tyr Phe Gln
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Pro Lys Pro Arg Gln Ala Trp Val Trp Ala Ala Val Arg Gly Pro
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Cys Ser Val Ser Cys Gly Ala Gly Leu Arg Trp Val Asn Tyr Ser
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Gln Gly Ser Gln Gln Pro Pro Ala Trp Pro Glu Ala Cys Val Leu
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Leu Gly Pro Gln Ala Gln Ala Pro Val Pro Ala Asp Phe Cys Gln
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His Leu Pro Lys Pro Val Thr Val Arg Gly Cys Trp Ala Gly Pro
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Cys Val Gly Gln Gly Thr Pro Ser Leu Val Pro His Glu Glu Ala
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Cys Arg Leu Phe Ile Asn Val Ala Pro His Ala Arg Ile Ala Ile
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Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
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Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
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Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
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Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu

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Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
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Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
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Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
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Glu Ile Gly His Ser Phe Gly Leu Glu His Asp Gly Ala Pro Gly
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Ser Gly Cys Gly Pro Ser Gly His Val Met Ala Ser Asp Gly Ala

170 175 180

gcg ccc cgc gcc ggc ctc gcc tgg tcc ccc tgc agc cgc cgg cag 585

Ala Pro Arg Ala Gly Leu Ala Trp Ser Pro Cys Ser Arg Arg Gln

185 190 195

ctg ctg agc ctg ctc agg acg ggc gcg ctg cgt gtg gga ccc gcc 630

Leu Leu Ser Leu Leu Arg Thr Gly Ala Leu Arg Val Gly Pro Ala

200 205 210

gcg gcc tca acc cgg gtc cgc ggg gca ccc gcc gga tgc gca gcc 675

Ala Ala Ser Thr Arg Val Arg Gly Ala Pro Ala Gly Cys Ala Ala

215 220 225

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Trp Pro Leu Leu Gln Arg Gln Arg Ala Val Pro Arg Gly Leu Arg

230 235 240

ccc caa ggc tgt cgc ctg cac ctt cgc cag gga gca cct gga tat 765

Pro Gln Gly Cys Arg Leu His Leu Arg Gln Gly Ala Pro Gly Tyr

245 250 255

gtg cca ggc cct ctc ctg cca cac aga ccc gct gga cca aag cag 810

Val Pro Gly Pro Leu Leu Pro His Arg Pro Ala Gly Pro Lys Gln

260 265 270

ctg cag ccg cct cct cgt tcc tct cct gga tgg gac aga atg tgg 855

Leu Gln Pro Pro Pro Arg Ser Ser Pro Gly Trp Asp Arg Met Trp

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Arg Gly Glu Val Val Leu Gln Gly Ser Leu Pro Leu Pro Gly Gly

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Pro Asp Val Phe Gln Ala His Gln Glu Asp Thr Glu Arg Tyr Val
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ctc acc aac ctc aac atc ggg gca gaa ctg ctt cgg gac ccg tcc 135
Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser
35 40 45

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Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
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Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr Ser
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Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
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Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
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Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
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Glu Ile Gly His Ser Phe Gly Leu Glu His Asp Gly Ala Pro Gly
155 160 165

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170 175 180

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185 190 195

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Leu Ala Thr His Leu Cys Ala Gly Arg Ser Leu Ser Leu Gly Pro
215 220 225

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Ser Gln Glu Pro Ala Pro Gly Gly Gly Arg Gly Pro Arg Thr Pro
230 235 240

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Val Pro Thr His Lys Arg Pro Arg Phe Gln Thr Leu Pro Ser Ser
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Val Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro
35 40 45

Ser Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile
50 55 60

Leu Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr
65 70 75

Ser Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro
80 85 90

Glu Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile
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Thr Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg
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Gly Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys
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Leu Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala
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Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

Claims

1. A protease that is capable of cleaving a bond between residues Tyr-842 and Met-843 of von Willebrand factor (hereinafter referred to as "vWF") and comprises a polypeptide chain having the amino acid sequence Leu-Leu-Val-Ala-Val as a partial sequence or an amino acid sequence with deletion, substitution, or addition of one or several amino acids in said amino acid sequence.

2. The protease according to claim 1, which comprises a polypeptide chain having the amino acid sequence Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val as the N-terminal partial sequence of a mature protein or an amino acid sequence with deletion, substitution, or addition of one or several amino acids in said amino acid sequence.

3. The protease according to claim 1 or 2, which comprises a polypeptide chain having an amino acid sequence with deletion, substitution, or addition of one or several amino acids in the amino acid sequence as shown in SEQ ID NO: 3 or 7 or a partial sequence of any of the aforementioned amino acid sequences as the N-terminal partial sequence of a mature protein or the aforementioned amino acid sequence.

4. The protease according to any one of claims 1 to 3, which comprises a polypeptide chain having an amino acid sequence with deletion, substitution, or addition of one or several amino acids in the amino acid sequence as shown in any of SEQ ID NOs: 16 to 21.

5. The protease according to any one of claims 1 to 4, which has molecular weight of 105 to 160 kDa or 160 to 250 kDa in SDS-PAGE under reducing or non-reducing conditions.

6. A gene fragment encoding a protease that is capable of cleaving a bond between residues Tyr-842 and Met-843 of vWF and comprises a polypeptide chain having the amino acid sequence Leu-Leu-Val-Ala-Val as a partial sequence or an amino acid sequence with deletion, substitution, or addition of one or several amino acids in said amino acid sequence.

7. A gene fragment encoding the protease according to any one of claims 2 to 5.

8. DNA encoding the protease according to any one of claims 1 to 5, which comprises a nucleotide sequence encoding a polypeptide capable of cleaving a bond between residues Tyr 842 and Met 843 of vWF comprising CTG CTG GTG GCC GTG or with deletion, substitution, or addition of one or several nucleotides therein.

9. The DNA encoding a protease according to claim 8, which comprises a nucleotide sequence comprising GCT GCA GGC GGC ATC CTA CAC CTG GAG CTG CTG GTG GCC GTG or with deletion, substitution, or addition of one or several nucleotides therein.

10. The DNA encoding a protease according to claim 8 or 9, which comprises a nucleotide sequence with deletion, substitution or addition of one or several nucleotides in the nucleotide sequence as shown in SEQ ID NO: 6 or a partial sequence of any of the nucleotide sequences, or the nucleotide sequence.

11. The DNA encoding a protease according to any one of claims 8 to 10, which comprises a nucleotide sequence with deletion, substitution or addition of one or several nucleotides in the nucleotide sequence as shown in SEQ ID NO: 15 or a partial sequence of any of the nucleotide sequences, or the nucleotide sequence.

12. A vector comprising the DNA encoding a protease according to claim 8 or 9, which comprises a nucleotide sequence with deletion, substitution or addition of one or several nucleotides in the nucleotide sequence as shown in SEQ ID NO: 6 or 15 or a partial sequence of any of the nucleotide sequences, or the nucleotide sequence.

13. The vector according to claim 12 comprising a polypeptide encoding domain and specialized in the expression of said polypeptide.

14. A cell transformed or transfected with the vector according to claim 12.

15. A host cell transformed or transfected with the expression vector according to claim 13.

16. A pharmaceutical composition comprising the protease according to any one of claims 1 to 5.

17. The pharmaceutical composition according to claim 16, which is applied to treating diseases caused by deterioration in activity of the protease according to any one of claims 1 to 5, which is involved with gene defects or liver diseases.

18. The pharmaceutical composition according to claim 16 or 17, which is applied to the inhibition of platelet aggregation caused by the formation of excess vWF high-molecular-weight multimers.

19. The pharmaceutical composition according to claim 18, wherein the disease is thrombotic thrombocytopenic purpura.

20. An antibody against the protease according to any one of claims 1 to 5.

21. The antibody according to claim 20 against the protease according to any one of claims 1 to 5, which is capable of inhibiting or neutralizing the protease activity.

22. The antibody according to claim 20 against the protease according to any one of claims 1 to 5, which can be used for affinity purification of the protease.

23. A process for purifying the protease according to any one of claims 1 to 5, which utilizes the antibody according to claim 22.

24. A pharmaceutical composition or diagnostic agent comprising an antibody against the protease according to any one of claims 1 to 5.

25. An antagonist, inhibitor, agonist, or activity regulator against the protease according to any one of claims 1 to 5.

26. A pharmaceutical composition or diagnostic agent comprising an antagonist, inhibitor, agonist, or activity regulator against the protease according to any one of claims 1 to 5.

27. A pharmaceutical composition or diagnostic agent comprising the DNA according to any one of claims 8 to 11 or antisense DNA thereof.

28. The pharmaceutical composition according to claim 27, which is used for gene therapy intended to cure diseases caused by deterioration in activity of the protease according to any one of claims 1 to 5, which is involved with gene defects or liver diseases.

29. A process for assaying vWF-cleaving activity, wherein a protease-substrate reaction is carried out using vWF and vWF-cleaving protease on a membrane filter, and a substrate sample is then recovered from the filter, followed by SDS-PAGE analysis without Western blotting.

30. A process for screening for a compound capable of cleaving vWF, wherein the vWF-cleaving activity of a test

compound is assayed by the process according to claim 29.

31. A process for preparing the protease according to any one of claims 1 to 5, wherein human plasma fraction I paste is used as a starting material.

32. A homologue of the protease according to any one of claims 1 to 5 derived from a different animal species or a homologous protein thereof.

33. A gene encoding the homologue of the protein according to claim 32 derived from a different animal species or a homologous protein thereof.

34. An animal having a modified gene encoding the homologue of the protein according to claim 32 derived from a different animal species or a homologous protein thereof.

FIG 1

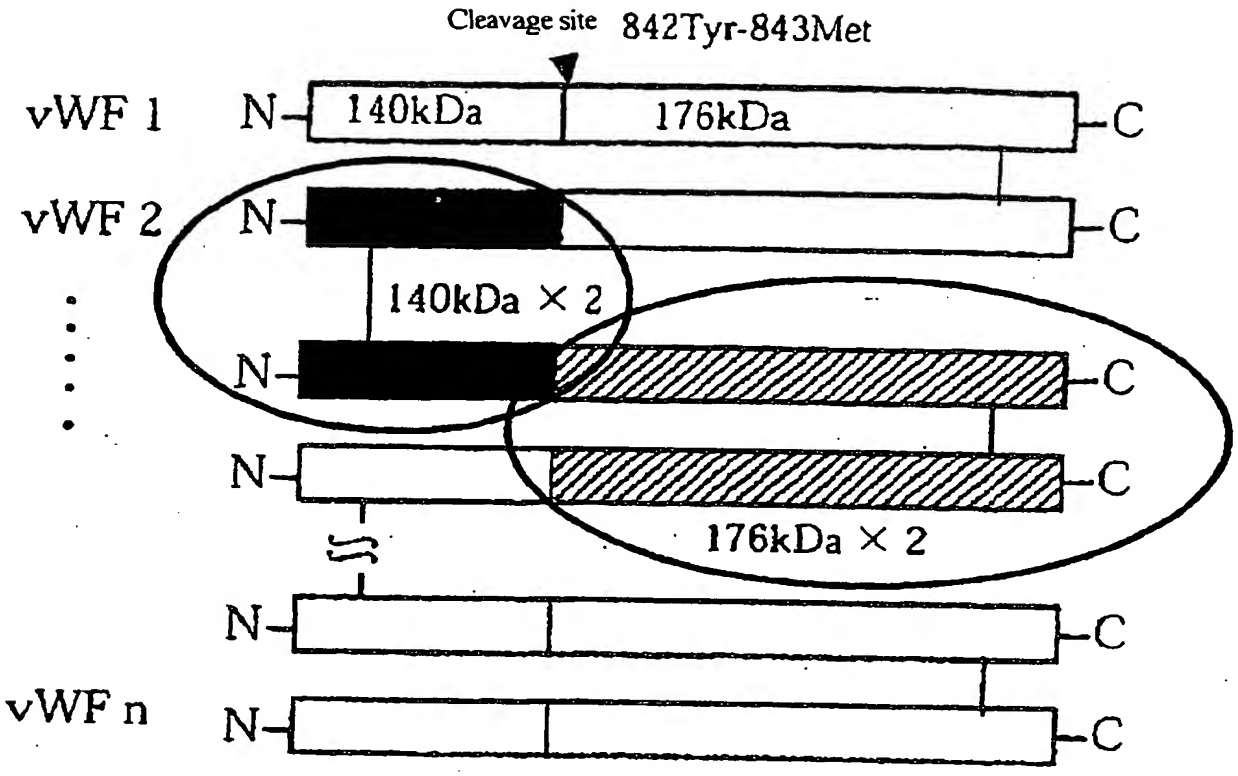

FIG 2

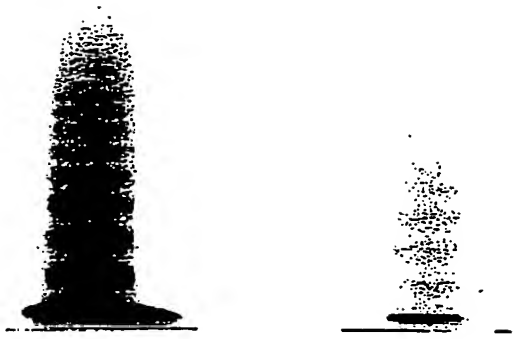

FIG. 3

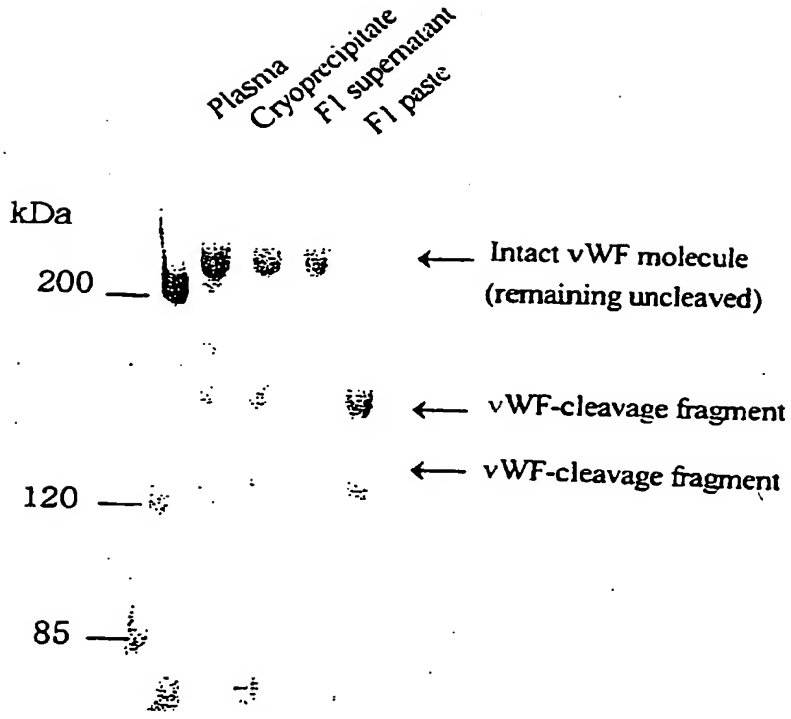

FIG. 4

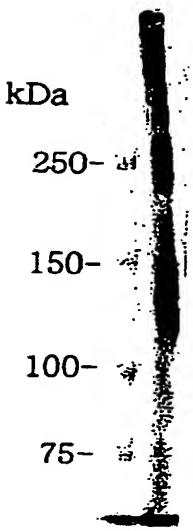

FIG. 5A

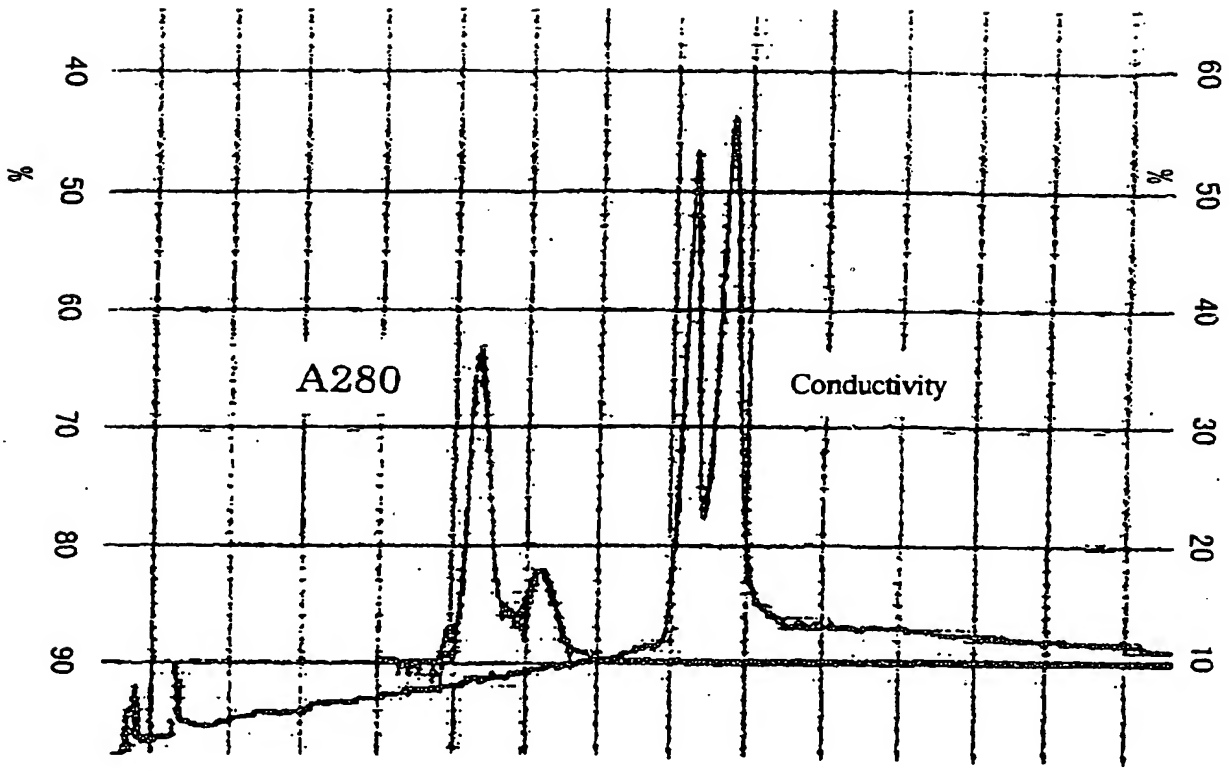

FIG 5B

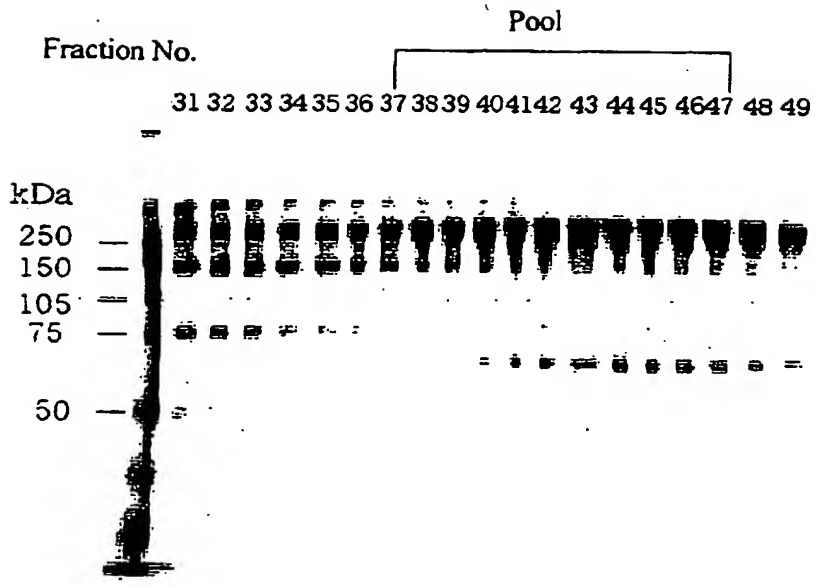

FIG 5C

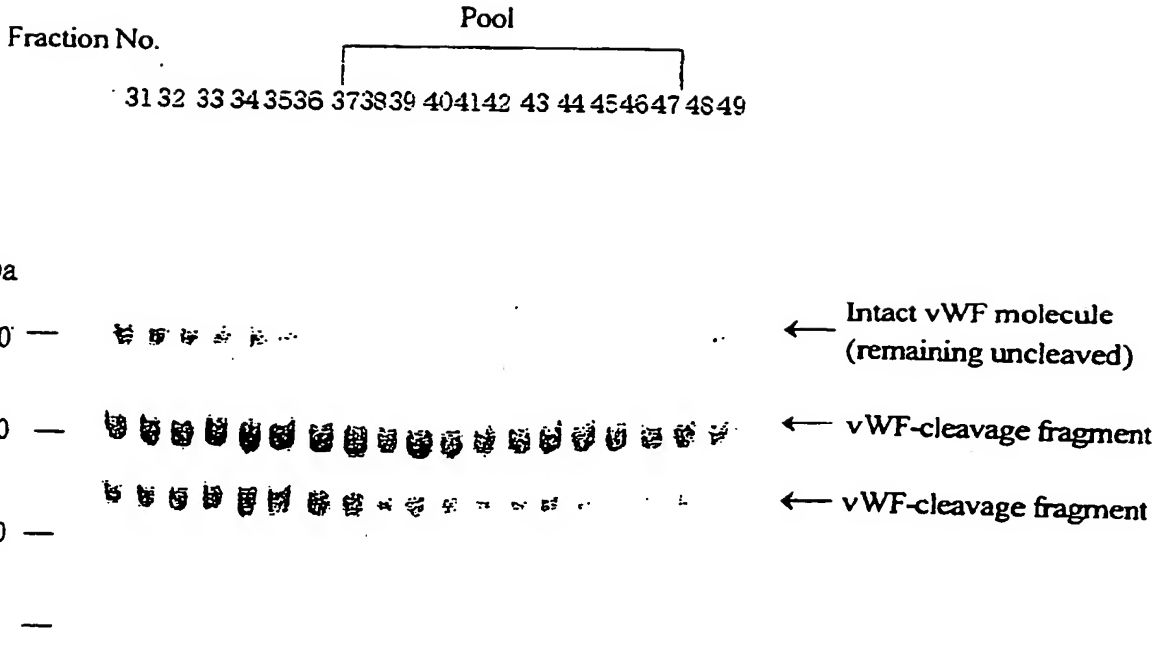

FIG 6A

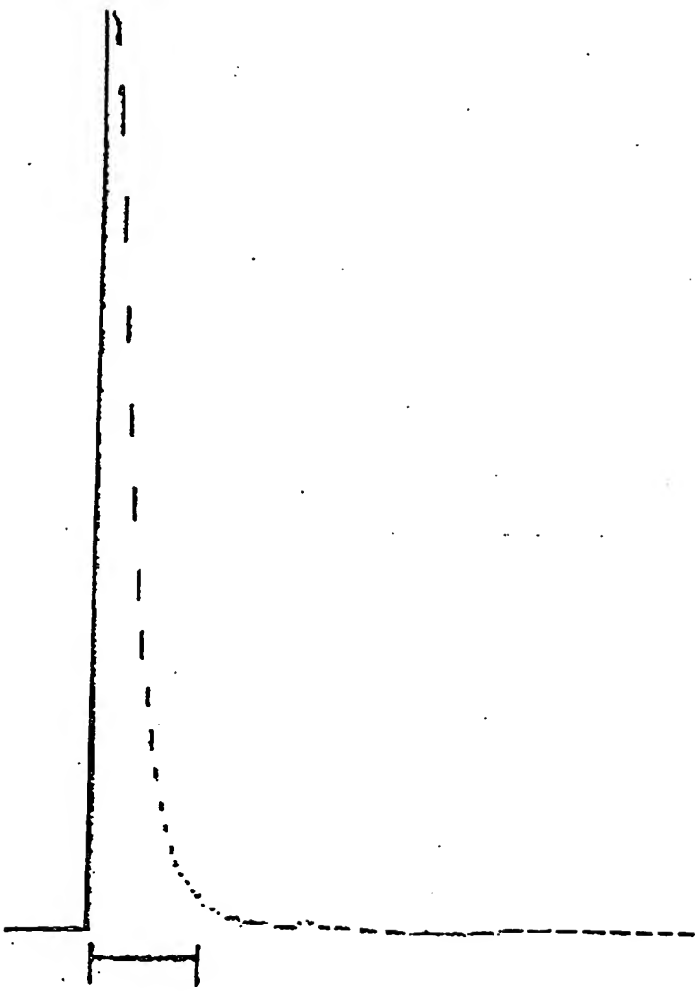

FIG 6B

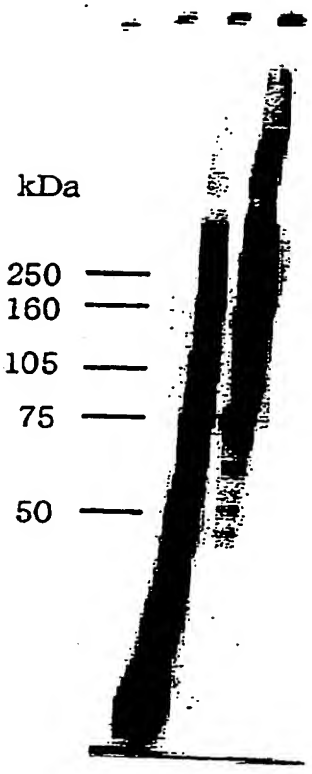

FIG 6C

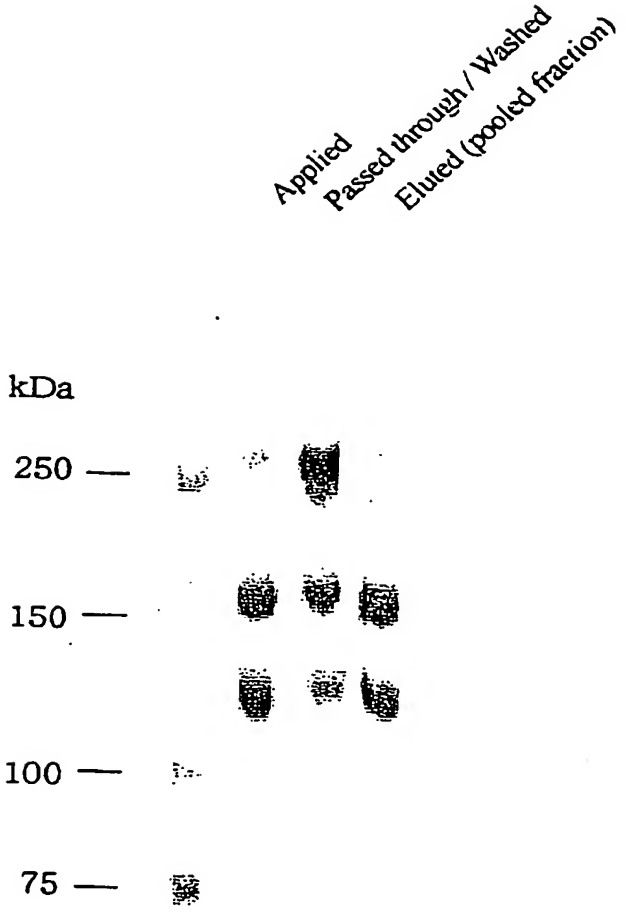

FIG. 7

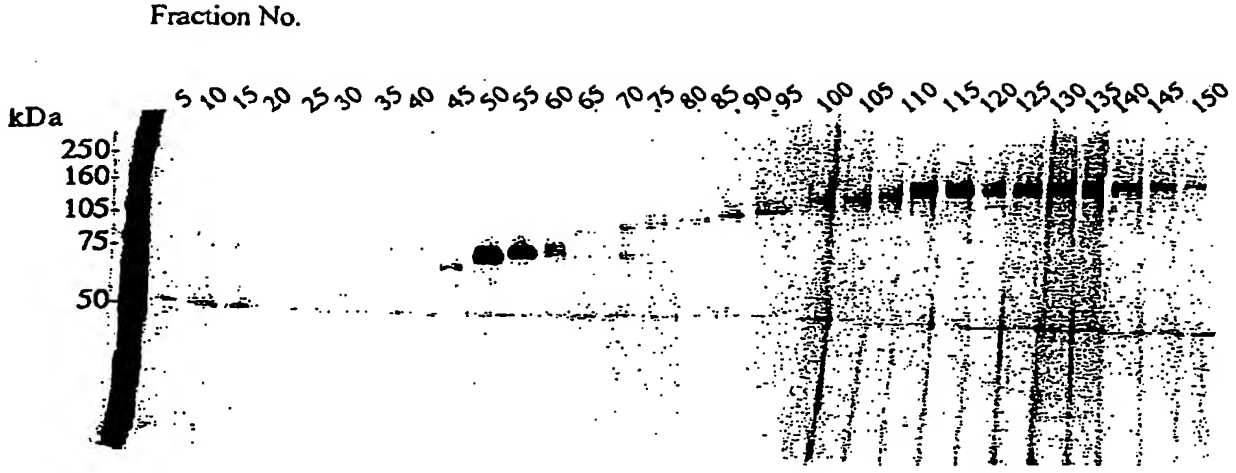

FIG 8A

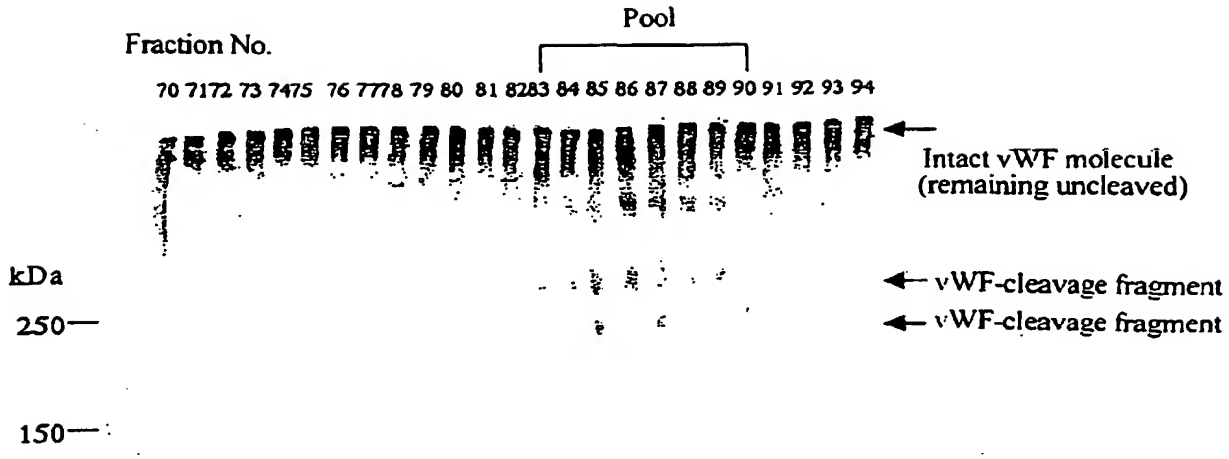

FIG. 8B

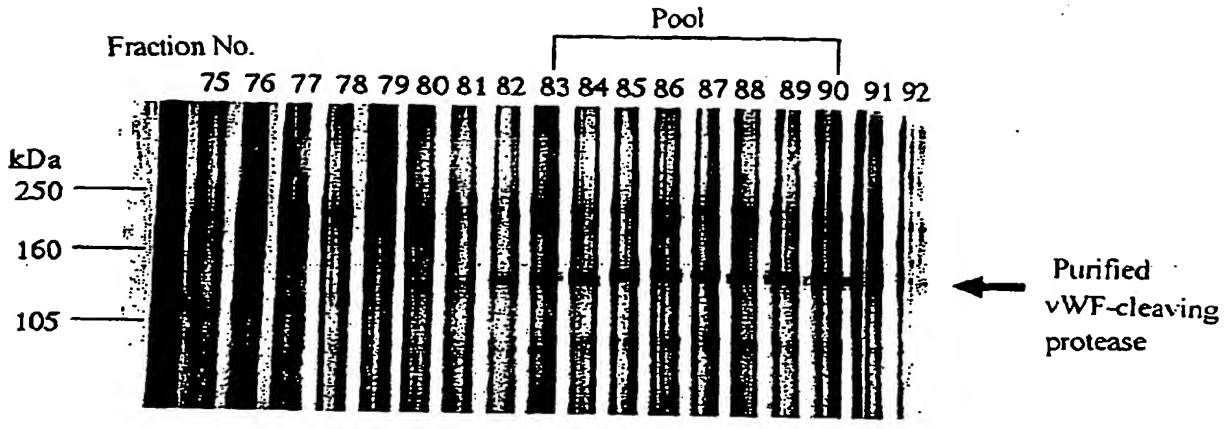

FIG 9

```
gct gca ggc ggc atc cta cac ctg gag ctg ctg gtg gcc gtg ggc
Ala Ala Gly Gly Ile Leu His Leu Glu Leu Leu Val Ala Val Gly
1               5                  10                  15
ccc gat gtc ttc cag gct cac cag aag gac aca gag cgc tat gtg
Pro Asp Val Phe Gln Ala His Gln Lys Asp Thr Glu Arg Tyr Val
            20                  25                  30
ctc acc aac ctc aac atc ggg gca gaa ctg ctt cgg gac ccg tcc
Leu Thr Asn Leu Asn Ile Gly Ala Glu Leu Leu Arg Asp Pro Ser
        35                  40                  45
ctg ggg gct cag ttt cgg gtg cac ctg gtg aag atg gtc att ctg
Leu Gly Ala Gln Phe Arg Val His Leu Val Lys Met Val Ile Leu
    50                  55                  60
aca gag cct gag ggt gct cca aat atc aca gca aac ctc acc tcg
Thr Glu Pro Glu Gly Ala Pro Asn Ile Thr Ala Asn Leu Thr Ser
65                  70                  75
tcc ctg ctg agc gtc tgt ggg tgg agc cag acc atc aac cct gag
Ser Leu Leu Ser Val Cys Gly Trp Ser Gln Thr Ile Asn Pro Glu
            80                  85                  90
gac gac acg gat cct ggc cat gct gac ctg gtc ctc tat atc act
Asp Asp Thr Asp Pro Gly His Ala Asp Leu Val Leu Tyr Ile Thr
        95                  100                 105
agg ttt gac ctg gag ttg cct gat ggt aac cgg cag gtg cgg ggc
Arg Phe Asp Leu Glu Leu Pro Asp Gly Asn Arg Gln Val Arg Gly
    110                 115                 120
gtc acc cag ctg ggc ggt gcc tgc tcc cca acc tgg agc tgc ctc
Val Thr Gln Leu Gly Gly Ala Cys Ser Pro Thr Trp Ser Cys Leu
125                 130                 135
att acc gag gac act ggc ttc gac ctg gga gtc acc att gcc cat
Ile Thr Glu Asp Thr Gly Phe Asp Leu Gly Val Thr Ile Ala His
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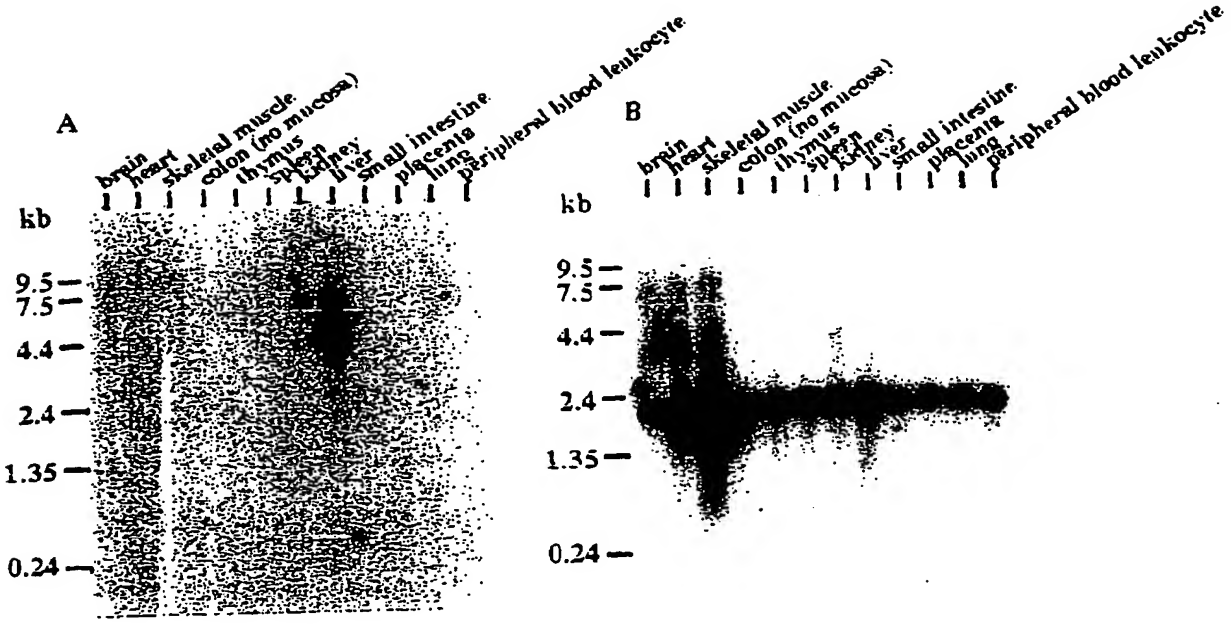
FIG. 10



brain	kidney
heart	liver
skeletal muscle	small intestine
colon (no mucosa)	placenta
thymus	lung
spleen	peripheral blood leukocyte

FIG. 11

gctgcaggcg gcatcctaca cctggagctg ctggtggccg tgggccccga tgtcttccag
Primer 1
gctcaccaga aggacacaga gcgctatgtg ctcaccaacc tcaacatcgg ggcagaactg
Primer 3
cttcgggacc cgtccctggg ggctcagttt cgggtgcacc tggtgaagat ggtcattctg

acagagcctg agggtgctcc aaatatcaca gcaaacctca cctcgtccct gctgagcgtc

tgtgggtgga gccagaccat caaccctgag gacgacacgg atcctggcca tgctgacctg
Primer 4
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ggcttcgacc tgggagtcac cattgcccat gagattgggc acagcttcgg cctggagcac
Primer 2
gac

Primer 1
Sense: gctgcaggcg gcatcctaca cctggagctg
Antisense : cagctccagg tgtaggatgc cgcctgcagc

Primer 2
Sense : accattgccc atgagattgg g
Antisense : cccaatctca tgggcaatgg t

Primer 3
Sense : gcgctatgtg ctcaccaacc tcaacatcgg
Antisense : ccgatgttga ggttggtgag cacatagcgc

Primer 4
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Antisense : gtgtcgtcct cagggttgat

FIG. 12

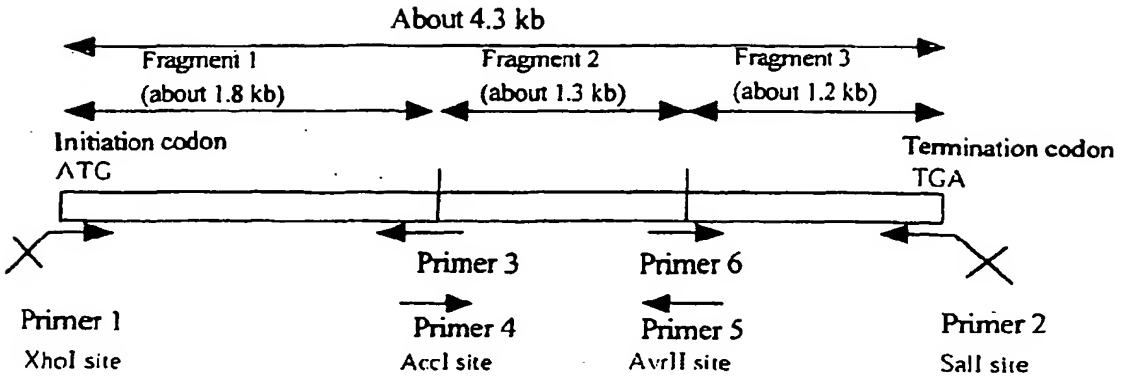


FIG. 13

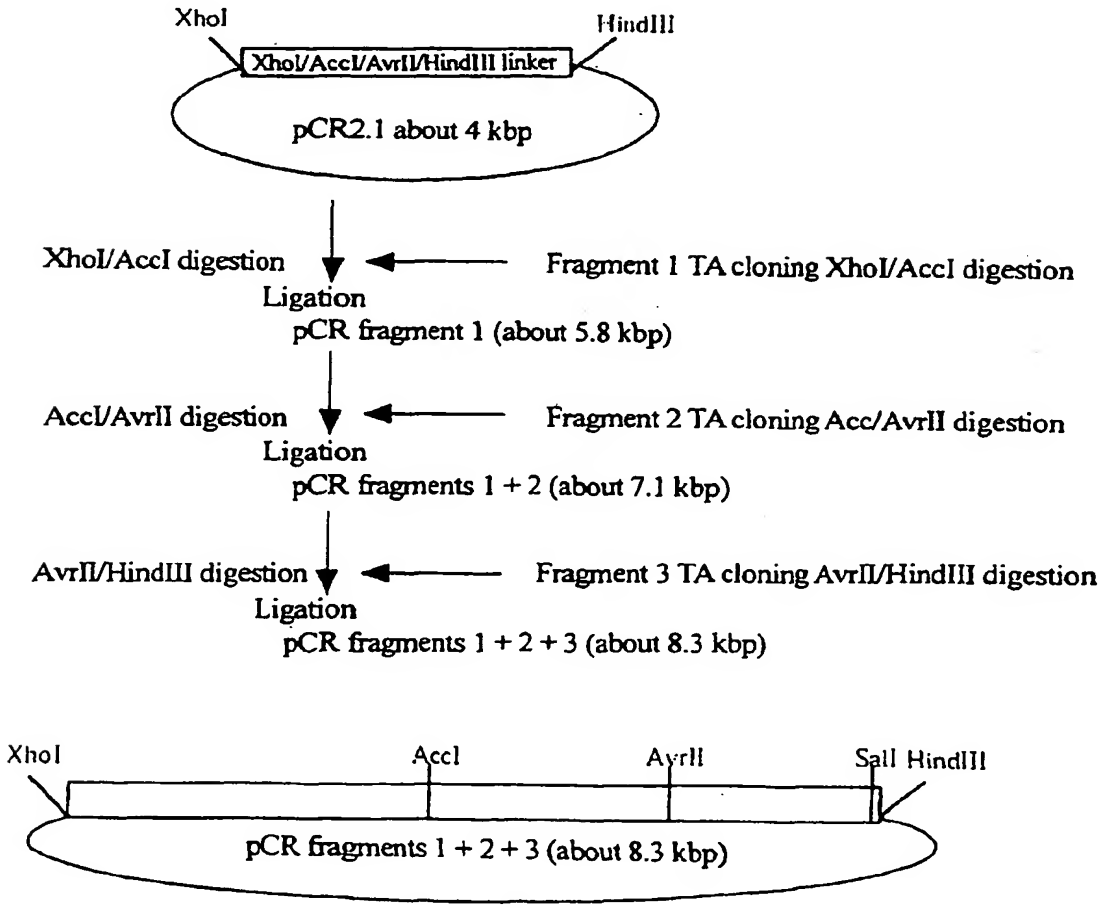


FIG 14

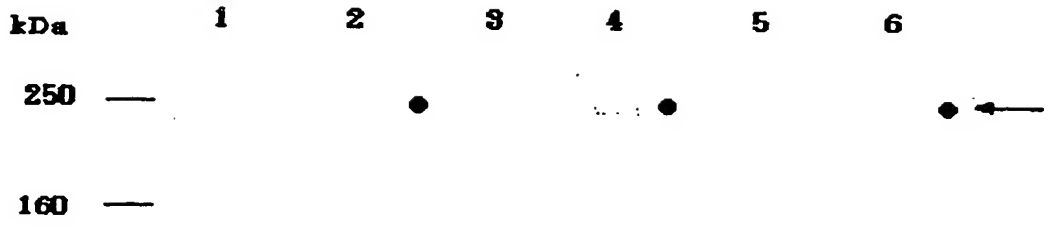


FIG 15

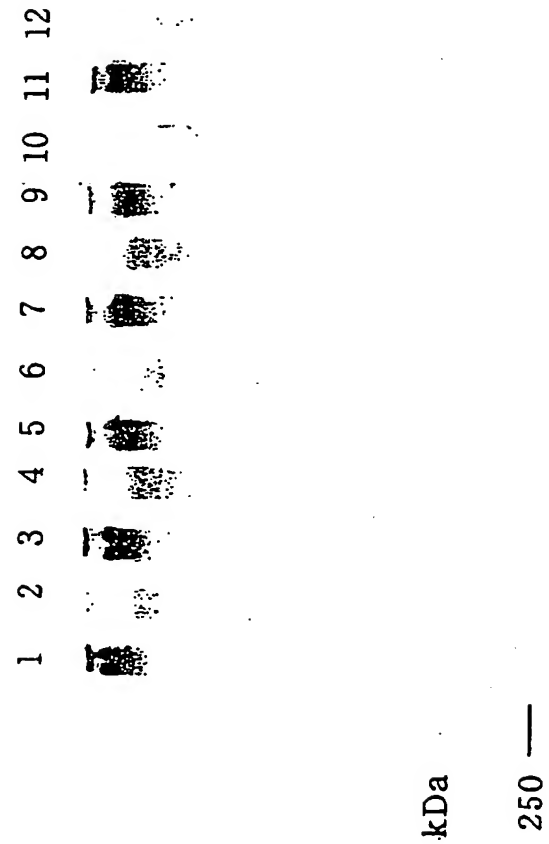


FIG 16

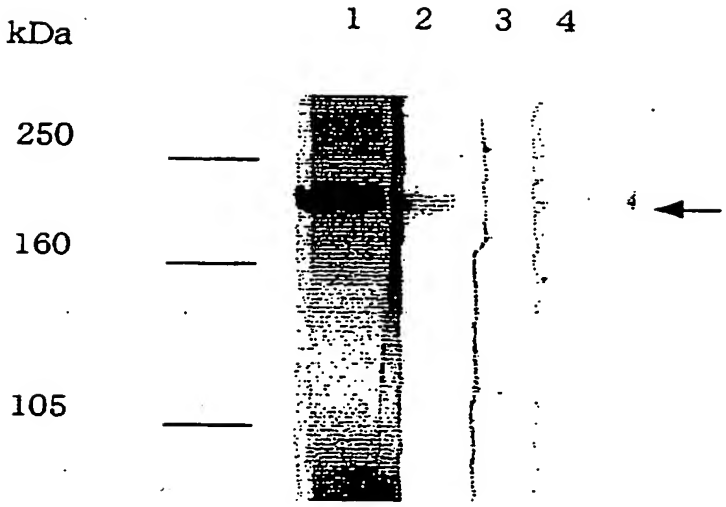


FIG. 17

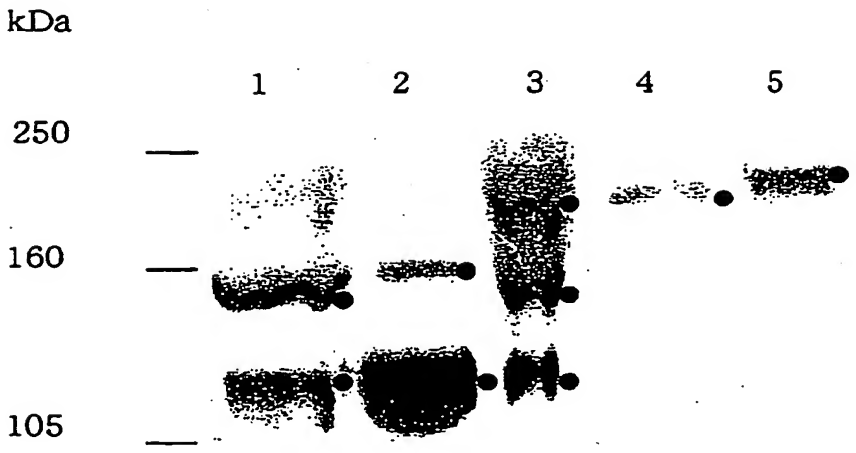


FIG. 18

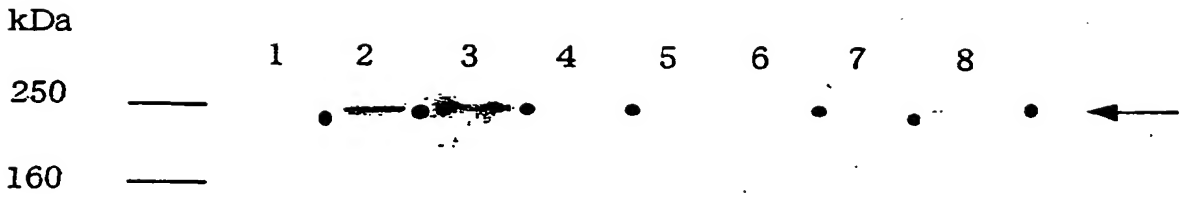


FIG. 19

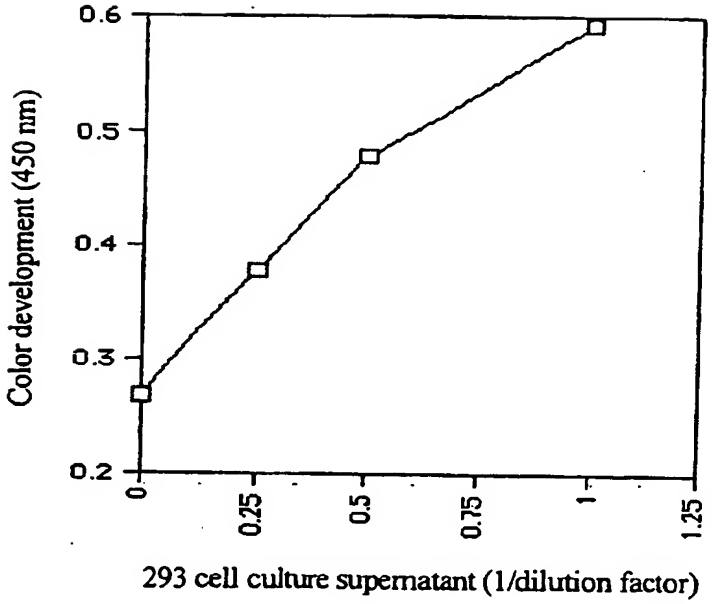


FIG. 20

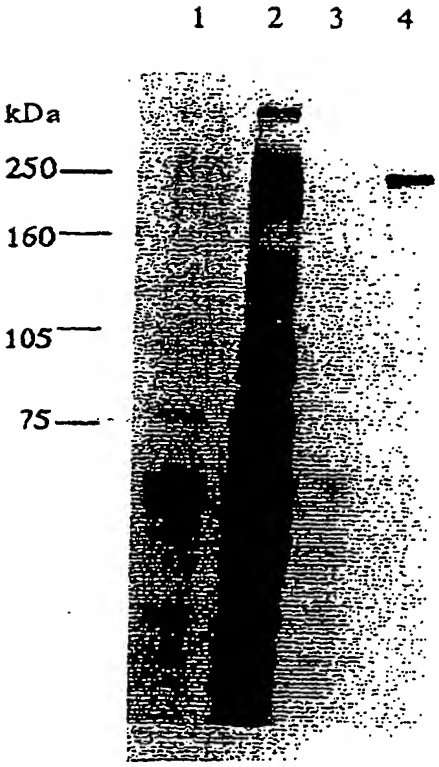


FIG 21

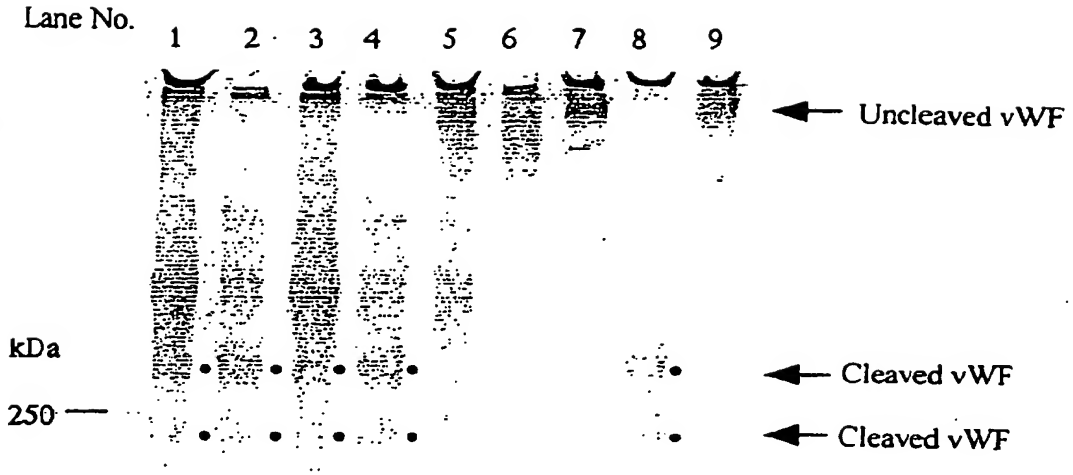
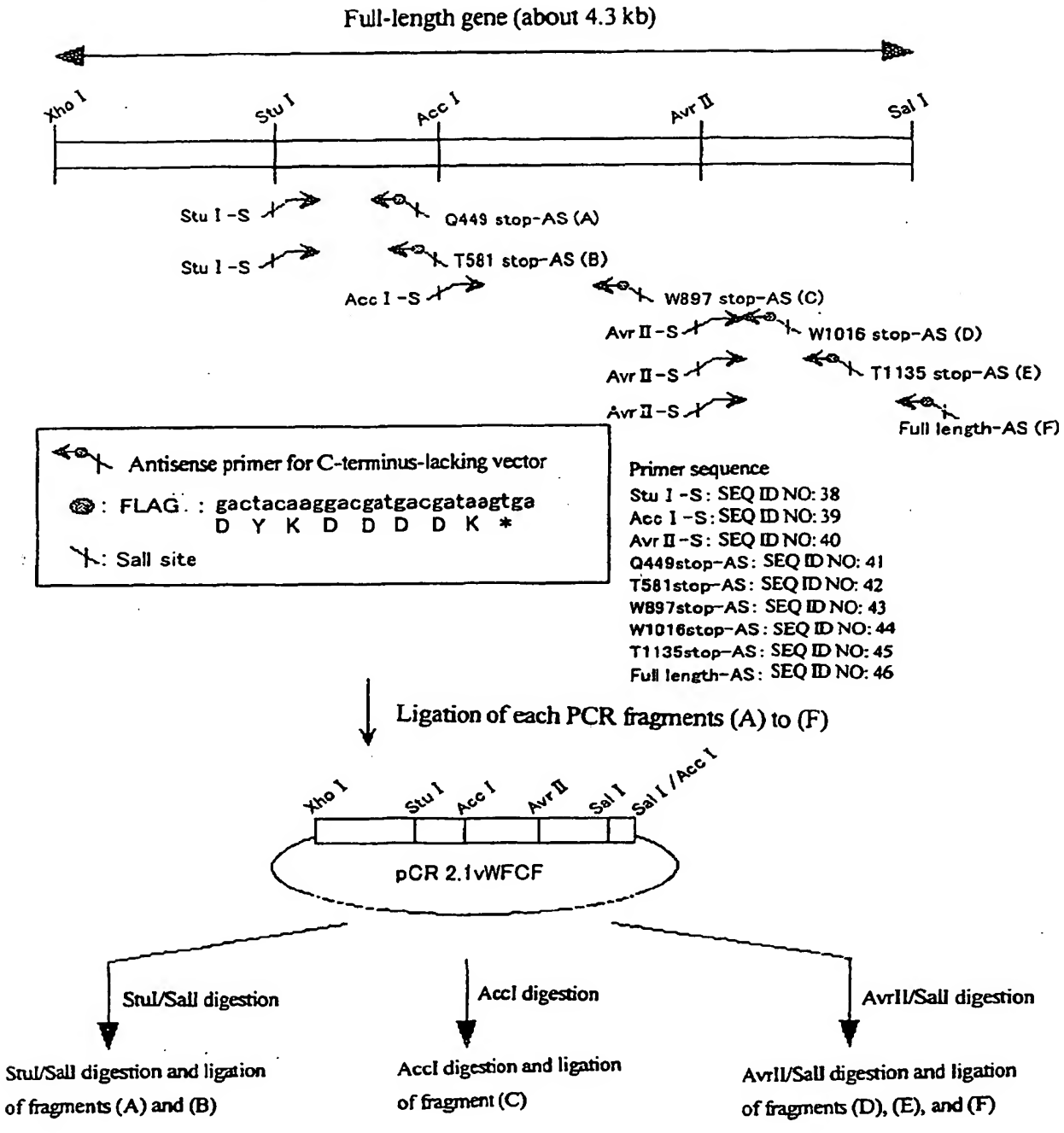


FIG 22



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/04141

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N15/57, C12N9/50, C12P21/00, A01K67/027, C12N1/15, C12N1/19, C12N1/21, C12N15/00, A61K38/46, A61P7/02, A61P43/00, A61K45/00, A61K48/00, A61K31/711, G01N33/573.A, G01N33/573.Z,
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] C12N15/00-15/57, C12N9/50, A61K38/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
SwissProt/PIR/GeneSeq, GenBank/EMBL/DDBJ/GeneSeq, BIOSIS(DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y/A	JP 2000-508918 A (Immuno AG.), 18 July, 2000 (18.07.00), & WO 97/41206 A3	1-5,20-27, 29-32/ 16-19/6-15, 28,33,34
Y/A	Miha FURLAN et al., Acquired Deficiency of von Willebrand Factor-Cleaving Protease in a Patient With Thrombotic Thtombocytopenic Purpura., Blood, 15 April, 1998 (15.04.98), Vol.91, No.8, pages 2839 to 2846	16-19/ 1-15,20-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" eadier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June, 2002 (14.06.02)	02 July, 2002 (02.07.02)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/04141

Continuation of A. CLASSIFICATION OF SUBJECT MATTER
(International Patent Classification (IPC))

Int.Cl[7] G01N33/15.Z, G01N33/50.Z

(According to International Patent Classification (IPC) or to both national classification and IPC)

Form PCT/ISA/210 (extra sheet) (July 1998)